A Rapid Screening Technique for Determining the Lipid Composition of Soybean Seeds

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An efficient method to determine the fatty acid composition of a large number of small seed tissue samples is needed to facilitate the genetic manipulation of seed lipid composition in soybean and other oilseeds. A rapid procedure has been developed to simultaneously extract and transmethylate the neutral lipids from soybean cotyledons at 90°C in a mixture of hexane and 1% H_2SO_4 in methanol (pretreatment of cotyledon pieces with hexane prior to methanolysis was found to improve yields). Fatty acid methyl esters were analyzed by capillary or packed column gas chromatography. The method required less than 10 mg of cotyledon tissue which could be taken from opposite the embryo axis so the sampled seed remained viable.

This direct transmethylation method was compared to Soxhlet extraction and subsequent acid methanolysis and to methods requiring separate steps for extraction [with hexane alone, hexane:isopropanol (3:2) or chloroform:methanol (2:1)], washing, solvent removal and methanolysis. Determinations of fatty acid content and composition were similar among the four methods. Approximately 30 times more samples could be analyzed (more than 2000 samples per month) using the direct transesterification method than were possible with the Soxhlet procedure. This direct transmethylation method is more rapid, requires less tissue, and provides results comparable to other inexpensive methods. It should be especially useful in breeding studies where large numbers of small samples are encountered.

The fatty acid composition of soybean (*Glycine max* (L.) Merr.) oil is the most important factor affecting oil oxidative stability (1,2). Efforts to improve the quality of soybean and other oilseed lipids through genetic manipulation have progressed, but large numbers of samples need to be analyzed (3-8). Thus, the ability to rapidly and accurately screen large numbers of seed samples for lipid composition is essential to efficient screening for variants (4).

The AOAC-accepted reflux-extraction procedures, such as Goldfisch and Soxhlet, are not suitable for the large number of small samples encountered in breeding programs. Currently, most screening methods use separate extraction, saponification and esterification steps prior to analysis of the fatty acid esters by gas chromatography. These multi-step procedures are time consuming and often expose the analyst to potentially hazardous solvent mixtures and reagents (9-11). Some of the screening methods also require larger quantites of tissue than can be obtained nondestructively from individual seeds.

Chaven et al. (12) described a relatively simple technique for isolating the neutral lipid fraction of soybean lipids and analyzing their fatty acid composition. The microanalytical procedure described by Chaven et al. (12) was suitable for small samples (ca. 50 mg) but still required separate steps for extraction, filtration, acid-methanolysis, solvent removal and sample dilution. Also, a separate seed sample (ca. 1 g) was needed to estimate oil content by nuclear magnetic resonance (NMR). Reports of direct transesterification of plant leaf (13) and animal tissues (14) suggested that it should be possible to perform these steps simultaneously in a single tube.

We have modified the microanalytical technique to allow simultaneous estimation of lipid content (mg lipid/mg tissue dry weight) and composition (mol% of the major fatty acids) by direct transmethylation of soybean lipids and subsequent analysis of the fatty acid methyl esters by capillary gas chromatography. The extraction and the methanolysis steps were carried out in inexpensive, disposable vessels. This report describes our modification of the microanalytical procedure and a systematic comparison of this method with others using the exhaustive soxhlet extraction method and other multiple-step solvent system extraction methods.

MATERIALS AND METHODS

Butylated hydroxytoluene (BHT), anhydrous Na_2SO_4, fatty acid and fatty acid methyl ester standards were obtained from Sigma Chemical Co. (St. Louis, Missouri). Methanol, chloroform, hexane, isopropanol, acetone, glacial acetic acid, petroleum ether (b.p. 40-60°C) and sulfuric acid were ACS grade (Fisher, Cincinnati, Ohio). A stainless steel gas chromatography column (183 cm × 3.2 mm) was packed with GP 3%, SP-2310/2% SP 2300 on 100/120 chromosorb WAW (Supelco, Inc., Bellefonte, Pennsylvania). A DB-Wax+ capillary GC column (30 m × 0.32 mm; 0.25 μm film thickness) was purchased from J & W Scientific, Inc.

Seed meal of *Glycine max* (L.) Merr., cv. McCall, was obtained by grinding mature seeds in a Braun coffee grinder. This seed meal was lyophilized and stored in a tightly sealed container at room temperature. Additional samples of McCall and four other genotypes were obtained by carefully removing ca. 10-25 mg fragments of cotyledon tissue distal to the embryo axis.

Microanalytical procedure. The procedure described here is that of Chaven et al. (12) and will be referred to throughout the text as the microanalytical method. Briefly, 50-100 mg seed meal was placed in glass test tubes (13 × 100 cm). Petroleum ether (5 ml; b.p. 40-60°C) containing 0.01% BHT was added to each tube. Samples were homogenized on ice and filtered into clean reaction tubes. Two ml H_2SO_4:methanol (1:99) along with a teflon boiling chip were added to each

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reaction tube. After vortexing briefly, the open tubes were placed in a heating block (90°C) for 10 min. Upon cooling, petroleum ether (3 ml; b.p. 40-60°C) was added to each tube. The tubes were vortexed for one min. The lower phase was removed and 0.2 g anhydrous Na_2SO_4 was added to the remaining organic phase. The samples were vortexed again, then centrifuged at 1000 g for five min. The clarified supernatants were transferred to serum vials and evaporated to dryness under N_2. These samples were redissolved in 50 μl of hexane with 0.01% BHT.

Direct transmethylation. Seed meal samples (routinely 5-25 mg) were weighed into tared 13 × 100 mm (disposable) borosilicate glass test tubes. Sample weight was recorded to the nearest 0.1 mg. To estimate methyl ester yield, heptadecanoic acid methyl ester (17:0me) was added as an internal standard. Where estimates of oil content (on a tissue dry weight basis) were desired, samples were spiked with 250 μg heptadecanoic acid (17:0) as the internal standard. Hexane (2 ml) containing 0.01% (w/v) BHT as an antioxidant was added to each sample along with a porous boiling stone. Two ml of H_2SO_4:methanol (1:99) were added to each sample tube. Samples were vortexed, then transferred to a heating block maintained at 90°C until the sample volume was reduced to 0.5 ml (about 20 min). Tubes were cooled to room temperature before 2.0 ml hexane with 0.01% BHT was added. The tubes were vortexed and anhydrous Na_2SO_4 (0.8—1.2 g) was added to each sample to remove any residual water. Samples were vortexed again. After allowing the samples to stand for ca. 30 min, the organic phase containing the fatty acid methyl esters (FAME) was transferred to Hewlett-Packard GC autosampling vials with teflon-coated septa and stored under N_2 at 5°C until analysis.

Soxhlet extraction (15,16). Five-g samples of the lyophilized seed meal (cv. McCall) were weighed into Whatman Cellulose extraction thimbles (single thickness, 33 mm i.d. × 118 mm external length) for Soxhlet extraction and gravimetric estimation of extractable lipid content. The thimbles were packed with glass wool and placed in a soxhlet extraction apparatus. Petroleum ether (b.p. 40-60°C) with 0.01% (w/v) BHT was added to each boiling flask with a few teflon boiling chips. The samples were refluxed (6-8 refluxes/hr) continuously for 48 hr with petroleum ether added as needed. Extracted lipids were rinsed with petroleum ether from the apparatus into preweighed beakers. The petroleum ether was allowed to evaporate until no petroleum ether odor was detected. Residual petroleum ether was removed *in vacuo*, and the oil content of the seed meal (w/w) was determined. A sample of the extracted oil (ca. 100 μl) was processed for Fame compositional analysis by sulphuric acid-catalyzed methanolysis as described above.

Multiple step solvent extraction/esterification method. Seed meal samples were weighed into glass tubes as in the microanalytical procedure. Lipids were extracted with 20 volumes of either chloroform:methanol (2:1, v/v) (17) or hexane:isopropanol, (3:2, v/v) (18). The extracts were filtered through sintered glass funnels, then washed with one-half volume of a 500 mM Na_2SO_4 solution to remove any remaining nonlipids. The organic phase was transferred to clean, dry tubes and evaporated to dryness under a stream of N_2 gas. The extracted lipid was resuspended in one ml of hexane with 0.01% (w/v) BHT. FAMEs for chromatographic analysis were again formed by sulphuric acid-catalyzed methanolysis.

Gas chromatographic analyses. Fatty acid composition of the lipid extracts was determined by temperature-programmed gas chromatographic analysis of the five major FAME components using either packed or capillary columns and a flame ionization detector.

FAME analysis with the packed column was performed as described by Wang et al. (19). A Hewlett-Packard model 5880A GC and terminal equipped with a 30 m × 0.322 mm DB-Wax+ column (0.25 μm film thickness) and a model 7673A autosampler was used for FAME analysis by capillary GC. A 1.0-3.0 μl aliquot was injected using the following conditions: operation in the splitless/split mode; inlet temperature 250°C; detector temperature 270°C; inlet purge flow 20 ml/min activated at one min. The column temperature was programmed at an initial temperature of 100°C for one min, then increased at 20°C/min to 200°C; column temperature was further increased at 4°C/min to 220°C and held there for five min. Helium was used as the carrier gas. There was no difference in the estimates obtained by the two methods, though the capillary column was more sensitive by about two orders of magnitude.

Peaks were identified by comparisons to retention of fatty acid methyl ester standards. Peak areas for palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic (18:3) fatty acids (taken as 100%) were obtained for each sample and identified by comparison to methyl ester standards. Myristic and palmitoleic acids were present in the extracts but represented <0.5% of the total in these tissues. Comparisons of the mean and variance for estimates of extractable neutral lipid (DW basis) and mol % of the five fatty acids were made using the SAS routines (20) for analysis of variance with balanced data sets (PROC ANOVA) and general linear model regression analysis for the unbalanced data sets (PROC GLM).

RESULTS AND DISCUSSION

Advantages of capillary column over packed-column. Capillary column GC offers a number of potential advantages over conventional packed column GC. Decreased run time per sample, reduced injection-to-injection variability, and increased sensitivity are among these. Running conditions for separation of the major FAMEs were optimized independently for the packed-column and capillary-column. Average retention times of the major FAMEs were calculated for both packed column and capillary column GC (Table 1). These estimates were based on three replicates of six one-μl injections of a mixture of FAME standards. Either method required about 15-20 min/sample.

Injection-to-injection variance was calculated to determine whether multiple injections of the same sample would be required. The coefficients of variation (CVs) between runs were 1.05% and 0.60% for the packed and capillary column systems, respectively (based on mol % composition values from six identical

TABLE 1

Comparison of Packed-Column and Capillary-Column GC[a]

Fatty acid methyl ester		Packed[b] column	Capillary[c] column
		Comparison of FAME elution profiles — Retention time (in min)	
Palmitic acid	C16:0me	6.15	7.60
Heptadecanoic acid	C17:0me	7.45	8.40
Stearic acid	C18:0me	8.80	9.25
Oleic acid	C18:1me	9.30	9.45
Linoleic acid	C18:2me	10.15	9.90
Linolenic acid	C18:3me	11.10	10.50

[a]Retention times based on average of six replicate injections of three FAME standard mixtures.
[b]The packed column was a 183 cm × 3.2 m stainless steel column and a 20 cm pre-column packed with GP 3%, SP-2300 on 100/120 Chromosorb WAW. See Materials and Methods for chromatographic details.
[c]The capillary column was a 30 m × 0.322 mm DB-Wax+ column (0.25 on film thickness). See Materials and Methods for chromatographic details.

TABLE 2

Response Correction Factors (RCF) for Converting FID Response to mol% and Coefficient of Variation (CV) of the Estimate

	Fatty acid methyl ester				
	C16:0	C18:0	C18:1	C18:2	C18:3
RCF[a]	0.97	1.03	1.01	0.98	1.04
CV (% of mean)	2.28	1.89	1.51	2.51	1.71

[a]None of the calculated RCF were significantly different than 1.00 by student's t-test ($\alpha = 0.05$).

injections of a mixture of Fame standards onto each column). While there was slightly less variation between runs with the capillary column, both methods gave reproducible estimates of FAME composition with very low injection-to-injection error. This suggests that a single injection of each sample should be sufficient using either system. The higher variability associated with the estimation on the packed column resulted from "peak tailing" of 18:3me at the higher temperature.

Increased sensitivity was the main advantage of using capillary GC. Accurate estimates of lipid composition were obtained with samples ranging from <10 ppm to >1000 ppm FAME using this capillary column. Along with the increased sensitivity came an increased possibility of error due to artifacts of the sample processing procedures. The 1% H_2SO_4 in methanol reagent used by Chaven et al. (12) originally was chosen over methanolic-HCl to avoid artifacts associated with the latter reagent (21). Either would probably give satisfactory results as methanolic-HCl has been used to directly transesterify leaf lipids (13). In each experiment, hexane (along and with 250 μg 17:0) was added to duplicate tubes and processed along with the tissue sample to indicate the presence of contaminant FAME in the standard or recurring artifacts due to the sample handling and processing procedures. There was no evidence of recurring artifacts that could interfere with the quantification of the major FAMEs or lipid content by capillary column GC.

The sensitivity of the capillary column system may also require special consideration when interpreting the data. It had been shown previously that chain length and degree of unsaturation affected FID response significantly (22) when capillary GC was used to quantify FAME. Therefore, response correction factors (RCFs) were determined experimentally to convert relative peak area to mol %. We calculated RCFs using mixtures of FAME standards (95% purity). None of the RCFs differed significantly from 1.0 (Table 2). Therefore, FID response was a reasonable approximation of mol % composition for these FAMEs. This observation might not have held true had FAME mixtures with a wider range of either chain length or degree of unsaturation been analyzed.

The major disadvantage of capillary versus packed columns is the much greater cost of replacing spent columns. However, we have been able to analyze more than 3000 samples with the same capillary column (described above) and have needed to remove only the first few meters of the column after about every thousand samples to regain the required resolution. We believe, therefore, that this disadvantage is more than offset by the advantages described above.

Optimization of sample size for reaction conditions. Samples for the microanalytical method (12) were routinely about 50 mg tissue dry weight. Though sufficiently small to allow nondestructive sampling of individual soybeans, that amount of tissue was too great to allow such sampling of some other oilseeds. Experiments were conducted to ascertain whether the 20–30 min reaction time at 90°C would be sufficient to accurately determine the lipid compositions of seed meal samples ranging from <5 mg to >250 mg dry weight. An internal standard, 17:0me, was added to each sample, and yield was calculated by comparing the observed 17:0me peak area to that expected if the seed meal contained 185 mg lipid g^{-1} tissue dry weight. The yield of methyl esters from crude seed meal samples decreased linearly as sample amount increased (Fig. 1). Yields were less than 70% for all samples over 80 mg seed meal. Yield could be increased with with higher temperature or longer reaction time; however, these changes frequently resulted in charring of samples in the open tubes.

There were only slight differences in the FAME composition estimates between samples, and these did not appear to be related to sample size. It was interesting that even in the largest sample, where the FAME yield was less than 30%, the composition estimates were consistent with the smaller samples. This would support the conclusion of Wiese and Snyder (23) that there is no unique, hard-to-extract lipid component of soybean meal, but rather an equilibrium rapidly occurs between solvent and tissue. When a simultaneous estimate of lipid content was desired, smaller samples (5–25 mg tissue dry weight) tended to give more accurate estimates as the external standard (17:0) appeared to be more "available" for transmethylation in the larger samples. This effect may be similar to the triglyceride adsorption phenomenon observed previously (23) in hexane extracts.

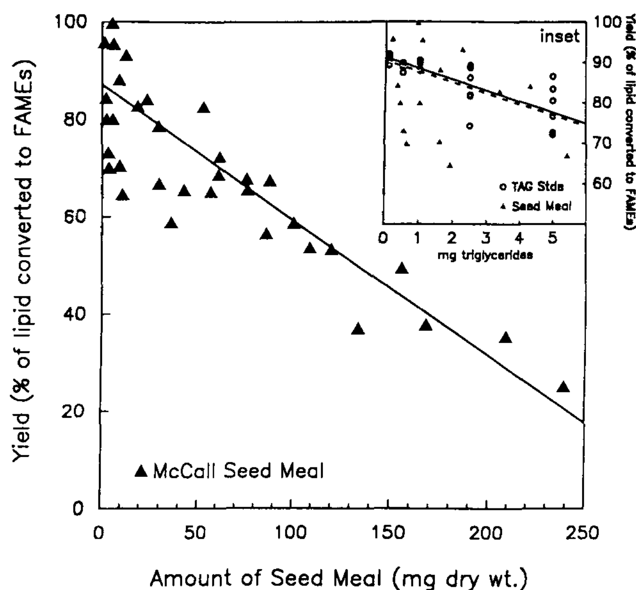

FIG. 1. Effect of increasing sample size on estimates of percent lipid. McCall seed meal samples were processed by direct methanolysis with 17:0me added as an internal control. Yield was calculated as percent of the seed oil that was converted to methyl esters. The oil content of the seed meal used in this study was determined by gravimetric analysis of soxhlet extracts to be 18.46. The data was fitted to a linear regression with slope = −2.78 (r^2 = 0.73). Inset: Effect of increasing triacylglycerol amount on FAME yield. Standard simple triglyceride mixtures were processed by the direct methanolysis method with 17:0me added as a control. Yield values (open circles) were calculated as percent of triglyceride added that was converted to fatty acid methyl esters. These values were fit to a linear regression (solid line) with slope = −2.74 (r^2 = 0.68). The observed yield from crude seed meal containing an equivalent amount of oil is indicated by the solid triangles and the fitted regression line (dashed line) with slope = −2.71 (r^2 = 0.08).

In the second series of experiments various combinations of the simple triglyceride standards, tripalmitin, tristearin, triolein, trilinolein and trilinolenin, were mixed with 17:0me and transmethylated in 1% H_2SO_4 in methanol. It was anticipated that this situation would be similar to that in the original microanalytical procedure (12). Again, the observed:expected ratio was used to calculate the methyl ester yield and accuracy of FAME composition estimates during the 20 min reaction time. Estimation of FAME composition from triglyceride standard mixtures was unaffected by increasing sample amount from 0.1 mg triglyceride to five mg triglyceride. These amounts would translate roughly to the amount of triglycerides present in 1-25 mg of soybean cotyledon tissue. The methyl ester yield from pure triglycerides decreases from 90% for 0.1 mg triglyceride to less than 70% for samples containing five mg triglyceride (Inset, Fig. 1). Though the r^2 value was low for the seed meal yields in this range, mean yield was consistent at each level with the yield observed for crude seed containing an equivalent amount of lipid. This suggests that there were no substances present in the crude soybean meal that could interfere with the acid-catalyzed methanolysis. Although alkaline-catalyzed methanolysis reactions proceeded much faster than sulphuric acid catalysis and at lower temperatures, crude seed samples contained substances that interfered with the alkaline esterification reactions (24). Because FAME composition estimates were unaffected by initial triglyceride content in either pure triglyceride or crude seed meal samples, the low yields were of no consequence for the compositional analyses.

Comparison of the direct transmethylation procedure to other methods of analysis. Ideally, the composition estimates obtained by any oilseed screening procedure would be representative of the commercially-extracted fraction (predominantly neutral lipids). Because the most commonly used commercial methods for soybean oil extraction use Hexane-type extractors, the process is readily simulated in the laboratory by a Soxhlet reflux-extraction apparatus (16). We compared the composition of hexane-extracted (Soxhlet) soybean oil to the values observed with the microanalytical and direct transmethylation methods. The composition estimates obtained from small samples (5-25 mg of seed meal) by the direct transmethylation procedure were similar, though not identical, to those obtained with an extensive Soxhlet extraction of large samples (>5 g of seed meal) (Table 3). The estimate obtained from mol % palmitic acid by the Soxhlet procedure was significantly lower than those obtained by three of the other methods. Values obtained using the microanalytical method for other fatty acids were not significantly different from the soxhlet or direct transmethylation methods.

The AOAC method for determining the oil content of soybean meal involves gravimetric analysis of the soxhlet extracts. Oil content was estimated with the direct transmethylation method by adding an internal standard, 17:0, to the seed samples prior to processing. We compared the oil content estimates obtained by these two methods. The oil content estimates obtained by adding the internal standard as the free fatty acid, 17:0, were also consistent with the Soxhlet procedure estimates if the tissue samples were finely ground (<40 mesh). This not only suggests the feasibility of simultaneously estimating content and composition, but also that the yields of FAMEs from triglycerides in the tissue and from externally applied fatty acids are relatively equivalent. The standard error for lipid content was much higher with the direct transmethylation method than with the Soxhlet method. This was probably the result of the extreme difference in sample size. Even if it were necessary to take multiple samples from the same population or the same individual, the direct transmethylation method would still represent a significant reduction in tissue and sample handling needs.

We also compared the direct transmethylation procedure to two other methods adapted to small sample size. The estimates of mol % from the direct method were consistent with the results of the chloroform:methanol and hexane:isopropanol methods for all fatty acids. It was concluded that although the direct transmethylation method gave slightly different results than the Soxhlet method, it was as accurate as the other screening methods tested.

Application of the direct transmethylation method to genotype screening. To test the feasibility of using

TABLE 3

Comparison of Composition and Content Estimates Obtained for Seed Meal Using Various Extraction Procedures

Method	Sample size tissue dry weight	Fatty acid composition (mol %) C16:0	C18:0	C18:1	C18:2	C18:3	Lipid content % of dry weight
Soxhlet (n = 18)	5-10 g	11.46[b] (0.66)	3.41[b] (0.22)	23.64[a] (3.79)	53.60[a] (3.26)	7.89[a] (0.66)	18.46[a]
Direct transmethylation (n = 16)	5-25 mg	15.22[a] (1.89)	4.18[ab] (0.53)	23.32[a] (3.86)	50.75[ab] (2.56)	7.53[ab] (1.30)	17.18[a] (2.06)
Microanalytical method (n = 30)	50-100 mg	12.91[ab] (2.01)	3.83[ab] (1.33)	24.23[a] (6.29)	51.17[ab] (5.29)	7.86[a] (2.49)	ND
Hexane:IPA (n = 8)	5-25 mg	14.63[a] (0.67)	4.51[a] (0.58)	25.08[a] (2.32)	49.23[b] (2.55)	6.55[b] (0.99)	ND
Chloroform:MeOH (n = 9)	5-25 mg	15.53[a] (1.11)	4.27[ab] (0.16)	23.79[a] (0.59)	49.41[b] (1.17)	6.99[ab] (1.52)	ND

[a]Samples from the microanalytical method and soxhlet extracts were analyzed using packed column GC. All other samples were analyzed using capillary column GC. Lipid content values were based on gravimetric and internal standard analyses for the soxhlet and direct transmethylation methods, respectively. Means and standard errors (in parentheses) are shown for each measurement. ND, not determined. Means within a column followed by the same letter are not significantly different by Fisher's LSD test (α = 0.05).

TABLE 4

Application of the Direct Transmethylation Method to Variant Screening

Genotype		Fatty acid composition (mol %) C16:0	C18:0	C18:1	C18:2	C18:3
McCall	mean	17.15[a]	3.72[b]	17.32[b]	53.11[c]	8.70[b]
	std dev	1.67	0.33	3.19	3.04	1.26
	range	15.03-20.80	3.38-4.50	12.98-22.19	49.60-58.00	6.65-9.93
Century	mean	15.58[b]	3.91[b]	10.05[d]	58.95[b]	11.52[a]
	std dev	1.08	0.33	3.81	2.52	2.58
	range	14.34-17.71	3.61-4.23	7.36-13.39	55.12-59.63	8.90-15.70
C1640	mean	15.55[b]	4.47[b]	12.11[cd]	62.86[a]	5.01[c]
	std dev	1.28	0.55	2.28	1.26	1.47
	range	14.09-17.42	3.84-5.49	7.14-15.53	61.48-64.67	3.41-5.83
A5	mean	16.10[ab]	3.38[b]	33.42[a]	42.57[d]	4.53[c]
	std dev	1.28	0.32	3.36	2.06	0.34
	range	14.65-18.40	2.99-4.02	28.30-37.14	39.42-45.35	4.01-4.96
A6	mean	12.70[c]	19.22[a]	13.30[c]	45.07[d]	9.71[b]
	std dev	1.12	5.52	1.97	3.66	1.61
	range	10.65-14.56	13.11-29.08	10.91-16.76	39.13-50.18	6.77-11.10

Means within a column followed by the same letter are not significantly different by Fisher's LSD test (α = 0.05).

Means, standard deviation and range. For each genotype, n = 9.

the direct transmethylation method for variant screening, samples of cotyledon tissue were removed from seeds of wild-type and variant soybean genotypes. McCall and Century were selected as wild-type cultivars representing high and low ranges for mol % 18:3 and 18:1. C1640 (6) and A5 (4) were independently identified low linolenic acid genotypes. The decrease in 18:3 was accompanied by an increase in 18:1 in the A5 genotype. A third variant, A6, was found to be high in stearic acid (4). The means and range of values observed (Table 4) for each of these genotypes obtained using the direct transmethylation method were in agreement with the earlier reports. It was possible to consistently identify the genotypes having abnormal lipid composition. While the method is not intended to replace established quantitative procedures, these results support the use of the direct transmethylation method for efficient screening for variation in soybean lipid composition.

We have found that FAME yields from soybean pieces (5-50 mg) average at least three times greater when an organic solvent such as hexane is added to the sample for at least 15 min prior to transesterification (data not shown). Some commercially available kits provide the means for very simple, efficient transesterification of fatty acids but at considerably increased

cost. The procedure described here should offer advantages over other accurate fatty analysis techniques where large numbers of samples need to be analyzed.

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