A Rapid Non-Destructive Technique for Fatty Acid Determination in Individual Peanut Seed[1]

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ABSTRACT

Modification of fatty acid composition can improve the keeping quality of peanut oil and may expand markets for peanut usage. Modification may involve hybridization to recombine existing genes, or the creation of new variability through mutagenesis or transformation with genes from other organisms. Identification of the fatty acid composition of individual seed could improve the chances of obtaining peanut genotypes with desired fatty acid composition. Published techniques for fatty acid analysis of individual peanut seed require the use of approximately half the cotyledonary seed tissue and utilize a process of solvent extraction and esterification. We have used a procedure that requires a small fraction of seed tissue and analyzes fatty acids through direct transmethylation.

Comparisons were made between procedures using seven genotypes representing a wide range of fatty acid composition variability. Quantities of fatty acids with C<20 were not statistically different between tests. While some differences were observed in longer-chained fatty acids (C≥20), the relative values among genotypes were similar. This procedure maintains greater integrity of the seed for planting purposes, requires less time and cost for fatty acid analysis, and can improve efficiency of individual seed analysis.

Key Words: analysis, *Arachis hypogaea* L., groundnut, lipids, oil, screening

Modification of the fatty acid composition in existing peanut cultivars can improve peanut products and may expand the utilization of peanut. This alteration of the composition may come from manipulation of existing variability, such as incorporation of the gene for high oleic/low linoleic acid (Moore and Knauft, 1989) from *Arachis hypogaea* L. or incorporation of genes for increased long chain fatty acid content from wild species (Stalker *et al.*, 1989). In other plant species, such as *Arabidopsis*, induced mutations have been used to produce lines with deficiencies in acyl chain elongation or with deficiencies in desaturation (Lemieux *et al.*, 1990). Recently Grayburn *et al.* (1992) transformed *Nicotiana tabacum* tissue with a rat stearyl-CoA desaturase gene. This transformation modified existing fatty acid variability and also caused synthesis of fatty acids not normally found in *Nicotiana* tissue. A similar procedure by Voelker *et al.* (1992) transformed *Arabidopsis* with a 12:0-acyl-carrier protein thioesterase from California bay (*Umbellularia californica*). The transformed *Arabidopsis* plants deposited laurate, a fatty acid normally found in only trace amounts, as the major fatty acid in storage triacylglycerols.

While induced mutations and transformation have not yet been used to modify fatty acid composition in peanut, these techniques, along with standard genetic procedures, can provide opportunities for increased variability in peanut seed tissue. A key to the successful utilization of any of these procedures is an efficient screening system. The published technique for individual seed analysis in peanut (Young and Waller, 1972) entails sampling the distal half of an individual seed, leaving the embryo half of the seed for grow out. That procedure also includes solvent extraction and esterification of the triacylglycerol, with subsequent fatty acid methyl ester (FAME) analysis using gas-liquid chromatography. In an attempt to decrease processing time of samples and reduce per sample cost, experiments were conducted to investigate the use of a new method for sampling cotyledonary tissue and a direct transmethylation procedure to prepare FAMES.

Materials and Methods

Two methods were compared to a control method normally employed in our laboratory for determining fatty acid composition of cotyledonary tissue from mature individual peanut seed (Table 1). Seven peanut genotypes with a range of fatty acid composition were used (Table 2). For the control group and method 2 group, half of a peanut seed, distal to the embryo, was chopped into a fine meal with a scalpel. Between 200 and 300 mg of the meal was placed into 13x100-mm culture tubes for the control samples. The method 2 samples were prepared by weighing 50 mg of chopped meal into 13x100-mm culture tubes. To test a faster sampling procedure a small coring device was constructed by sharpening a hollow tube 30-mm long by 2 mm in diameter (Fig. 1). Although several standard laboratory items may be appropriate for this device, we found it convenient to use a sports needle used for inflating basketballs and footballs. This device resulted in a sample core when driven through the peanut seed approximately 1 mm in diameter and 5 mm in length, with an average

Table 1. Summary of tissue sampling and fatty acid methyl ester preparation procedures employed for each of the experimental methods.

Method	Tissue sampling	FAMES preparation
Control	Chopped seed	Solvent extraction/esterification
Method 1	Core seed	Transmethylation
Method 2	Chopped seed	Transmethylation

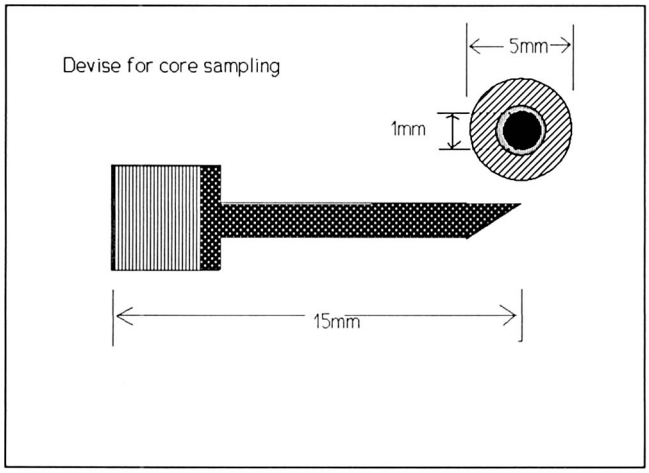

Fig. 1. Coring device used for sampling peanut seed.

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Table 2. Comparison of shorter chain fatty acid composition (C<20) of seven peanut genotypes analyzed with three sampling methods and FAMES preparation.

Genotype	Method	Fatty acid C16:0	C18:0	C18:1	C18:2	C18:3
		mol %				
F435	Control	6.57 (0.40)*	3.47 (0.46)	78.15 (0.90)	2.12 (0.58)	1.73 (0.18)
	Method 1	7.40 (0.33)	3.73 (0.86)	79.84 (1.16)	2.60 (0.20)	1.52 (0.23)
	Method 2	6.95 (0.31)	3.44 (0.38)	79.67 (0.76)	2.46 (0.33)	1.58 (0.12)
F78114B	Control	10.60 (1.40)	3.40 (0.88)	50.05 (6.70)	29.53 (5.97)	1.46 (0.21)
	Method 1	10.82 (0.90)	3.77 (0.58)	49.56 (2.91)	31.57 (2.57)	1.17 (0.14)
	Method 2	11.50 (1.33)	3.17 (0.68)	46.71 (6.01)	33.41 (5.46)	1.10 (0.19)
F78114W	Control	8.77 (0.85)	3.93 (0.68)	55.80 (4.79)	24.79 (4.03)	1.66 (0.11)
	Method 1	9.60 (1.34)	4.15 (1.21)	52.11 (6.26)	29.40 (6.09)	1.22 (0.22)
	Method 2	9.58 (1.24)	3.14 (0.52)	54.91 (7.29)	27.42 (6.17)	1.08 (0.12)
F640-B	Control	9.56 (0.60)	3.18 (0.43)	48.52 (3.80)	28.49 (3.65)	1.53 (0.18)
	Method 1	9.89 (0.98)	3.76 (0.44)	50.53 (5.36)	27.74 (4.21)	1.37 (0.11)
	Method 2	10.18 (0.48)	3.58 (0.52)	47.30 (3.48)	31.28 (3.14)	1.19 (0.15)
F640-R	Control	11.04 (1.34)	4.65 (0.71)	44.63 (3.42)	28.98 (4.58)	1.88 (0.30)
	Method 1	11.85 (1.04)	4.73 (0.53)	43.08 (1.82)	32.67 (3.21)	1.40 (0.17)
	Method 2	12.19 (1.15)	4.31 (0.76)	42.99 (3.58)	30.61 (2.58)	1.40 (0.17)
Florunner	Control	9.74 (0.93)	2.42 (0.35)	54.49 (5.32)	25.97 (4.34)	1.29 (0.12)
	Method 1	9.96 (0.89)	2.57 (0.48)	57.24 (4.84)	25.04 (4.32)	1.09 (0.16)
	Method 2	10.30 (0.95)	2.24 (0.33)	53.83 (5.57)	28.15 (5.11)	1.01 (0.10)
F81206	Control	7.27 (0.65)	3.52 (0.44)	65.21 (4.45)	15.77 (3.97)	1.71 (0.12)
	Method 1	7.36 (0.85)	3.78 (0.59)	67.05 (4.61)	14.94 (4.13)	1.40 (0.15)
	Method 2	7.85 (0.65)	3.20 (0.35)	65.13 (4.51)	17.36 (4.04)	1.35 (0.12)

* Means and standard errors (in parentheses) are shown for each measurement.

weight of 15 mg. This core was placed in 13x100-mm culture tubes for the samples of method 1.

It has been shown by Kartha (1963) that tissue samples from the middle section of peanut seed gave optimal representation for fatty acid composition and iodine value. This was confirmed by our lab (data unpublished), and samples taken by the chopping method involved removing a small portion of the distal end and chopping the remaining distal half. In the core sampling for method 1, the core was removed from the mid section of the seed perpendicular to the plane of the cotyledons. This caused very little disruption of seed integrity, leaving a very small 1 mm-diameter hole through the middle of the seed.

In preparing FAMES for the control group, the solvent extraction/esterification protocol modified from Mozingo *et al.* (1986) was used. A brief summary of this method follows.

1. 200-300 mg of chopped sample were placed in 13x100-mm culture tubes, 2 mL of petroleum ether was added, the tubes were sealed with Teflon-lined caps, vortexed, and allowed to stand overnight.
2. The next day the sample tubes were centrifuged, and the supernatant pipetted into 16x125-mm culture tubes and evaporated under a stream of N_2.
3. 2 mL of 0.5M NaOH-methanol was added, tubes were vortexed and heated in 100C water bath for 5 minutes.
4. After tubes cooled, 2 mL of boron trifluoride-methanol (14%) was added, tubes were vortexed and then heated in 100C water bath for 5 minutes. Tubes were cooled and 2 mL of water was added to stop the reaction.
5. 2 mL of petroleum ether was added to the tubes. After a thorough mixing and phase separation, 1.5 mL of the upper phase (FAMES) was removed by pipette into 2 mL Hewlett-Packard auto sampler vials.

Because of success with direct transmethylation procedures used in our lab for fatty acid analysis of leaf tissue and because of the potential for decreasing sample time, the direct transmethylation methods of Browse *et al.* (1986) were employed to test the feasibility of using this procedure in high oil tissue. A summary of this procedure is as follows.

1. To either the 15-mg core sample or the 50-mg chopped sample of Method 1 or Method 2, respectively, 1 mL of 1M methanolic HCl with 5% (v/v) 2,2-dimethoxypropane was added to sample tubes that were filled with N_2 and sealed with Teflon-lined caps.
2. The sample tubes were placed in 80 C water bath and heated for 1 hour.
3. After cooling the tubes, 1 mL of 0.9% NaCl was added to stop the reaction. 1.5 mL of hexane was added, the solution was thoroughly mixed, the phases were allowed to separate, and 1 mL of the upper phase (FAMES) was pipetted into Hewlett-Packard autosampler vials.

All samples from the method 1 and 2 and the control group were analyzed by a Hewlett-Packard model 5890A gas chromatograph. FAMES were separated on a 6' x 1/8"-glass column packed with SP-2330 and 100/120 WAW Chromosorb (Supelco Inc.) and measured with a flame ionization detector. Injector and detector temperatures were set at 250 C. Oven temperature was programmed for an initial temperature setting of 190 C for 3 minutes, then increasing at a rate of 3 C per minute until reaching a final temperature of 220 C. Integration of peak areas and percent fatty acid composition calculations were determined by a Hewlett-Packard 3392A integrator. Identities of fatty acids were established by comparison to standard.

Results and Discussion

The data from the experiments are summarized in Tables 2 and 3. The transmethylation procedure used in methods 1 and 2 gave results similar to the control method. This is particularly evident for the shorter chain fatty acids (less than 20 carbons) present in quantities greater than 2% of the total fatty acid composition. For palmitic (16:0), stearic (18:0), oleic (18:1), and linoleic (18:2) acids no statistical differences were found between means of the control group and methods 1 or method 2. At this time these fatty acids, especially oleic and linoleic, are of most interest to peanut breeders for introduction of the high oleic trait into breeding lines. Direct transmethylation for FAMES preparation is an acceptable and accurate procedure for determining fatty acid composition of the shorter chain fatty acids (C<20).

When the proportion of a particular fatty acid is low in a sample, results of analysis from direct transmethylation differ slightly from those of the solvent extraction/transesterification procedure. Although representative of relative presence of these fatty acids, the results show significant differences between fatty acid composition means (Tables 2 and 3). As noted by Dahmer *et al.* (1989), the direct transmethylation procedure is not a substitute for the more rigorous analytical solvent extraction/esterification methods commonly employed, but it is an extremely useful and time

Table 3. Comparison of long chain fatty acid composition (C>18) of seven peanut genotypes analyzed with three procedures for sampling and fatty acid methyl ester preparation.

Genotype	Method	Fatty acid C20:0	C22:1	C24:0
		mol %		
F435	Control	2.62 (0.13)*	2.66 (0.31)	1.72 (0.15)
	Method 1	1.27 (0.15)	2.40 (0.22)	1.03 (0.11)
	Method 2	1.52 (0.14)	2.89 (0.28)	1.27 (0.14)
F78114B	Control	0.95 (0.19)	2.65 (0.47)	1.37 (0.19)
	Method 1	0.62 (0.06)	1.69 (0.14)	0.74 (0.10)
	Method 2	0.73 (0.16)	1.92 (0.46)	0.89 (0.30)
F78114W	Control	0.96 (0.08)	2.68 (0.22)	1.33 (0.12)
	Method 1	0.64 (0.06)	1.78 (0.14)	0.80 (0.07)
	Method 2	0.83 (0.13)	1.74 (0.07)	0.78 (0.08)
F640B	Control	1.10 (0.17)	3.29 (0.63)	1.64 (0.32)
	Method 1	0.82 (0.12)	2.11 (0.33)	2.50 (0.83)
	Method 2	0.85 (0.17)	1.99 (0.81)	1.85 (0.57)
F640R	Control	0.74 (0.09)	2.77 (1.00)	2.35 (1.15)
	Method 1	0.60 (0.07)	2.10 (0.51)	2.30 (1.70)
	Method 2	0.67 (0.07)	2.04 (0.35)	2.88 (1.13)
Florunner	Control	1.26 (0.14)	2.70 (0.23)	1.57 (0.15)
	Method 1	0.98 (0.14)	1.81 (0.35)	0.96 (0.18)
	Method 2	1.09 (0.12)	1.87 (0.15)	0.98 (0.11)
F81206	Control	1.34 (0.11)	3.65 (0.38)	1.54 (0.11)
	Method 1	1.02 (0.07)	2.48 (0.31)	0.94 (0.12)
	Method 2	1.17 (0.09)	2.60 (0.28)	1.00 (0.10)

* Means and standard errors (in parentheses) are shown for each measurement.

saving procedure for first estimation of fatty acid composition for cultivar screening.

One of the attributes of the direct transmethylation procedure is the ability to use small samples. FAMES composition estimates are optimized at sample sizes less than 100 mg (Dahmer *et al.*, 1989, and Browse *et al.*, 1986). Our procedures can exploit this utilization of small sample size with a rapid seed coring technique that produced a tissue sample of approximately 15 mg and an intact seed that germinated readily. The results from the core samples analyzed as method 1 and the results from chopped samples analyzed as method 2 (Tables 2 and 3) show no significant differences between them for the eight fatty acids under study. Therefore, the coring method of tissue preparation is an acceptable method for cotyledonary tissue sampling.

The time saving in tissue preparation of the coring method over chopping the seed is dramatic and allows for more samples to be processed in a given day. On average (averages computed on 150 samples, data not shown) it took half the time to core a seed than it did to chop. Elimination of the hazard of cuts from a scalpel is also a significant advantage to this coring method.

Further time savings are gained by using the transmethylation method over the solvent extraction/esterification method. Not counting the 12 hour solvent extraction, but timing the steps of saponification, esterification, and extraction it takes approximately twice as long per sample for the solvent extraction/esterification method as it does for the direct transmethylation procedure. With the coring/direct transmethylation method a large number of samples can be prepared in one day and analyzed overnight on a gas chromatograph with autosampler, thus greatly reducing processing time.

Another important consideration in large scale analysis is cost. The current cost of materials and reagents for the solvent extraction/esterification procedure are slightly more than twice the cost of the coring/direct transmethylation procedure. An additional cost savings occurs with the reduced labor requirements of the coring/direct transmethylation procedure. A final benefit is the smaller amount of solvent needed for the direct procedure, which can reduce potential environmental hazards associated with solvent removal, as well as cost of disposal.

This technique will provide peanut researchers with the opportunity to determine fatty acid composition of individual peanut seed. The technique is more rapid and less expensive than conventional procedures, and it maintains the integrity of individual seed. Results from the standard technique (Young and Waller, 1972) and this rapid procedure varied only for longer chain fatty acids (C>18), normally present as less than 4% of the total fatty acid composition.

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