# Microsomal Polypeptide Comparisons Between High and Normal Oleic Acid Isogenic Peanut Lines Using Two-Dimensional Gel Electrophoresis[1]

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**ABSTRACT**

Peanut (*Arachis hypogaea* L. subsp. *fastigiata* var. *vulgaris*) developing seed microsomal polypeptides of the high oleic acid variant (F435) and its presumed isogenic, normal line (78-1339) were compared using two-dimensional gel electrophoresis. A pair of 20 kDa polypeptides focusing at pH 6.8 and 7.3 was present in all of the polypeptide profiles from both isolines regardless of maturity or genotype except for (F435) at stage 1 maturity. The stage 1 (F435) profile contained, instead, an 18 kDa polypeptide pair focusing at about pH 9.3. Based on correlation evidence, we postulate that the 20 kDa polypeptides could be components of the $\Delta^{12}$-desaturase complex. The 18 kDa polypeptides appeared to be associated with lower desaturase activity, reduced linoleic acid and increased oleic acid seed content. Since the 18 kDa polypeptides focusing at pH 9.3 are not found at later stages, they are probably under developmental control. Changes in the developing seed polypeptides of the microsomal fraction over the four maturities are also reported.

Key Words: Peanut, 2D-gel electrophoresis, microsomal polypeptide, desaturase.

Peanut (*Arachis hypogaea* L.) is a major food crop. Worldwide it is used for oil and food products. In the United States, peanut consumption is mainly roasted nuts, peanut butter and candy. Whether used for oil or nuts, the quality of the product depends on the properties of the oil. Fatty acid composition is important among the oil characteristics. Of the 12 fatty acids detected in peanuts, palmitic acid (16:0), oleic acid (18:1) and linoleic acid (18:2) predominate. Those three make up over 90% of the fatty acid composition in peanut (Norden *et al.*, 1987) with oleic acid and linoleic acid making up about 80%. Saturated fatty acids, such as palmitic acid, are chemically stable relative to unsaturated fatty acids. However, their inclusion in human diets has been associated with high serum cholesterol and heart disease, and they are considered undesirable for health reasons. Healthful polyunsaturated fatty acids such as linoleic acid lack stability. Oxidation of polyunsaturated fatty acid produces acids, ketones and other hydrocarbons that result in unpleasant rancid flavors and odors that shorten the shelf life of peanut products. Polyunsaturated fatty acids in peanut oil also cause more rapid breakdown under cooking heat than saturated and monounsaturated oils. Oleic acid is more stable than linoleic acid and has been found to be as healthful as polyunsaturated fatty acids (Grundy, 1986). Therefore, it is desirable to maximize the oleic acid content in peanut.

In plants, fatty acid synthesis to stearic acid and the first desaturation of stearoyl-ACP to oleoyl-ACP by a soluble $\Delta^9$-desaturase occurs in the plastid. Oleoyl-ACP is exported from the plastid and converted to oleoly-CoA where it becomes part of the cytoplasmic acyl-CoA pool (McKeon and Stumpf, 1982). Further desaturation is catalyzed by an endoplasmic reticulum-bound $\Delta^{12}$-desaturase complex involving phosphatidylcholine as the carrier for the acyl chain, cytochrome b5 (Powell *et al.*, 1990) and other components. Regulation of that 18:2 desaturation is complex and Stymne *et al.* (1983) concluded that it depends upon 1) the selectivity, activity and rate of acyl exchange between the acyl-CoA pool and phosphatidylcholine, 2) the rate of desaturation of oleic acid in phosphatidylcholine, and 3) the activity, specificity and selectivity of glycerophosphate acyltransferase and diacylglycerol: acyl-CoA acyltransferase in moving acyl-CoA's from the pool into triglycerols and phospholipids.

A peanut line (F435) with very high oleic acid content (80% oleic and 2% linoleic acid) was reported by Norden *et al.* (1987). With the exception of this line, the oleic acid content of peanut germplasm ranges between 37% and 67%, and the linoleic acid content ranges between 15% and 43%. The high oleic acid line is believed to have originated in an individual plant (78-1339) which contained normal levels of fatty acids. The high oleic acid line and 78-1339 are believed to be isogenic except for the gene/s that caused the shift in oleic acid and linoleic acid concentrations. No differences have been noted between these two lines in other phenotypic characteristics including yield and market grade characteristics (D.A.K., unpublished). Moore and Knauft (1989) found that crosses using the high oleic acid line segregated either in 3:1 or 15:1 indicating recessive digenic regulation. Knauft *et al.* (1993) later reported that single gene recessive inheritance is the common mode of inheritance. Ray *et al.* (1993) demonstrated that the $\Delta^{12}$-desaturase activity was 10-fold lower in the high oleic acid peanut line than in its normal oleic acid isoline. The $\Delta^{12}$-desaturase activity was localized in the microsomes, and no changes in other enzyme activities associated with $\Delta^{12}$-desaturase were detected.

Since the site of $\Delta^{12}$-desaturation activity is in the microsomes and the high oleic acid line has reduced $\Delta^{12}$-desaturase activity, this research was conducted to determine whether polypeptide differences could be detected between the microsomal fractions of the high and normal oleic acid isolines over four stages of seed development. Microsomal polypeptide differences between the two isolines could provide clues to mechanisms causing the high oleic acid production. Two-dimensional gel electrophoretic analyses comparing the microsomal polypeptide composition of the high oleic acid (F435) and its normal oleic acid isoline (78-1339) during seed development are reported.

## Materials and Methods

**Plant Tissue:**

Developing seeds of the field-grown, high oleic acid peanut line (F435) and its isoline (78-1339) were harvested, graded into the four maturity stages as described in Table 1 and frozen in liquid nitrogen. Seeds were

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**Table 1. Distinguishing characteristics of the four simplified developmental seed stages used in this paper and their physiological maturity indices as described by Pattee *et al.* (1974).**

| Stage | Pod characteristic | Seed size (mg) | Seed coat color | Physiological maturity index |
|---|---|---|---|---|
| 1 | watery, soft & thick | 150 | white | 4 |
| 2 | soft & slightly thickened | 300 | pink at embryo end | 5, 6, 7 |
| 3 | dry, thin & papery inside | 600 | light pink all over | 8, 9, 10 |
| 4 | dry & indent inside | 600 | pink all over | 11, 12 |

stored at -70 C until needed.

**Microsomal Fractionation and Protein Isolation:**

The microsomal fraction of the developing seeds was isolated by a modification of the method of Slack *et al.* (1979). Frozen peanuts (2.5 g) were ground to a fine powder in liquid nitrogen using mortar and pestle. The frozen powder was mixed rapidly with 20 mL of microsomal isolation buffer (MI) consisting of 0.5 M Tris-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM MgCl, 10 mM cysteine, 0.33 M sorbitol, and protein stabilizers (0.2 g phenylmethyl sulfonylfloride and 0.1 g butylated hydroxytoluene per 200 mL of MI buffer). The extraction mixture was centrifuged at 18,000 x g for 10 min at 4 C. Both the pellet and floating fat were discarded and the aqueous phase was centrifuged for 1 hr at 105,000 x g. The membrane (microsomal) pellet was carefully rinsed with cold MI buffer which was then completely drained and blotted away. The pellet was resuspended in, and the membranes were solubilized with, 100 µL of sample loading/lysis buffer consisting of 9.5 M urea, 2% w/v of nonidet P-40 (NP-40), and 2% ampholytes (1.6% pH 5-8, and 0.4% pH 3-10) (Hurkman and Tanaka, 1986). Protein content was determined using the Bradford method after the samples were diluted to avoid interference from urea, ampholites and other loading/lysis buffer components. Samples were stored until needed at -70 C.

**First Dimension—Isoelectric Focusing:**

Isoelectric focusing (IEF) was conducted in tubes 1.5 mm inside dia and 14 cm long. Tubes were acid-cleaned and coated sequentially with Sigmacote (Sigma Chemical Co., St. Louis, MO) and Photoflow (Eastman Kodak Co., Rochester, NY) diluted according to the manufacturers' directions. IEF gels were prepared using 5.5 mL of a solution made as follows: 48.6 g urea, 28.8 mL deionized $H_2O$ ($dH_2O$), 11.8 mL of a 30% acrylamide/bis-acrylamide (37:1) solution, 20.3 mL 10% NP-40, 4.5 mL ampholytes pH 5-8, and 0.5 mL ampholytes pH 3-10. The upper and lower chamber buffers were reversed from the method of O'Farrell (1975), with the 0.06% phosphoric acid placed in the upper chamber and the 0.1 N NaOH placed in the lower chamber. All buffers were thoroughly vacuum degassed before using. Approximately 50 µL (50 mg) of protein were loaded per IEF gel. Isoelectric focusing was carried out at 400 V for 13 hr and then at 800 V for an additional 1.5 hr. Gels were removed from the glass tubes and placed directly into sodium dodecyl sulfate (SDS) equilibration buffer, consisting of 10% w/v glycerol, 2.3% w/v SDS, and 0.625 M Tris-HCl (pH 6.8), for 15 min according to the BioRad protocol. After exchanging the buffer with fresh buffer, the gels and buffer were frozen, and stored at -70 C until needed for the second dimension electrophoresis.

**Second Dimension—SDS Electrophoresis:**

Second dimension slab gels were poured and electrophoresed in pairs to compare the high and normal oleic acid lines in each run. Gels were 0.75 mm thick, with the separating gel being 12% acrylamide (33.5 mL $dH_2O$, 25 mL 1.5 M Tris-HCl [pH 8.8], 1.0 mL 10% w/v SDS, 40 mL 30% acrylamide/bis, 50 uL N, N, N', N'-tetramethylethylenediamine [TEMED]) and polymerized by 0.5 mL 10% ammonium persulfate. Stacking gels were 4% acrylamide (11.3 mL $dH_2O$, 5 mL 0.5 M Tris-HCl [pH 6.8], 0.2 mL 10% w/v SDS, 2.6 mL 30% acrylamide/bis, 20 µL TEMED) and were polymerized with light after adding 1 mL riboflavin (4 mg/100 mL). The equilibrated IEF gels were thawed and then fixed to the top of the stacking gel with 1% agarose in stacking gel electrophoresis buffer.

The two gels were electrophoresed at 40 mA for 4.5 to 5 hrs or until the bromphenol blue running dye was within 1 cm of the bottom of the gel. The gels were removed, fixed in 40% methanol:10% acetic acid, and silver stained twice according to the Bio-Rad silver stain protocol to intensify staining sensitivity. BioRad SDS low molecular weight protein markers were used to estimate polypeptide sizes. Gels were photographed and examined for polypeptide differences between high and normal oleic acid lines and among peanut maturity stages.

## Results and Discussion

The 9 M urea, 4% NP-40 and 2% ampholyte sample loading/lysis buffer solubilized the microsomal pellet well, could be loaded directly on the IEF gel, and gave very good IEF resolution. Preliminary experiments using buffers consisting of 50 mM Tris-HCl, 5% B-mercaptoethanol and 0.2% SDS or 10 mM CHAPS solubilized the microsomal pellet satisfactorily, but required additional preparation for IEF and resulted in streaking and poor resolution in the 2-D gel. Urea and ampholyte concentrations of the solubilization buffer were sufficient to cause interference with the Bradford protein assay. However, it was possible to dilute samples sufficiently to avoid that interference.

The two-dimensional gel polypeptide profiles of the microsomal fractions contained more than 250 polypeptides (see Figs. 1 and 2). The patterns of the high and normal oleic acid isolines were similar at each maturity stage. The gel profiles revealed polypeptide differences between the high and normal oleic acid lines, but consistent differences, over replicated gels, were observed only in the earliest developmental stage (stage 1). Those consistent differences involved a pair of polypeptides approximately 18 kDa in size and focusing at about pH 9.3 which was present in the high oleic acid line. The normal oleic line did not have that pair of polypeptides focusing at pH 9.3, but had a pair of polypeptides about 20 kDa in size and focusing at about pH 6.8 and 7.3. With the exception of the high oleic acid isoline at stage 1, the pair of 20 kDa polypeptides focusing at pH 6.8 and 7.3 was present in all of the other polypeptide profiles from both isolines regardless of maturity or genotype. The two pH 9.3 species are more closely grouped than those at pH 6.8 and 7.3. It is interesting to note that these polypeptides occur in pairs.

While we have no direct evidence that the two pairs of polypeptides are related, the presence of the 18 kDa pair in the high oleic line and the presence of the 20 kDa pair in the normal oleic acid line and their similar size suggests they may be modifications of similar polypeptides. However, a major modification would be required to cause the isoelectric point and size shift. Single point mutations which substitute one amino acid for another would probably not be sufficient to cause such a wide change. Mutations resulting in premature termination of transcription, such as read through or the introduction of new termination codons and post translational modification could cause the observed shift. Anderson and Anderson (1979) demonstrated that proteins or living cells could be chemically treated to block protein glycosylation that would change the relative gel positions of those proteins when electrophoresed. They also observed that such treatment could affect the number of isozymic forms found. Since Powell *et al.* (1990) found that the high oleic trait was not expressed in shoots and leaves of the high oleic acid genotype and postulated that the $\Delta^{12}$-desaturase system was under spacial and temporal regulation, the lesion causing the high oleate trait could well be in that regulatory system.

The similar polypeptide patterns of the two isolines show

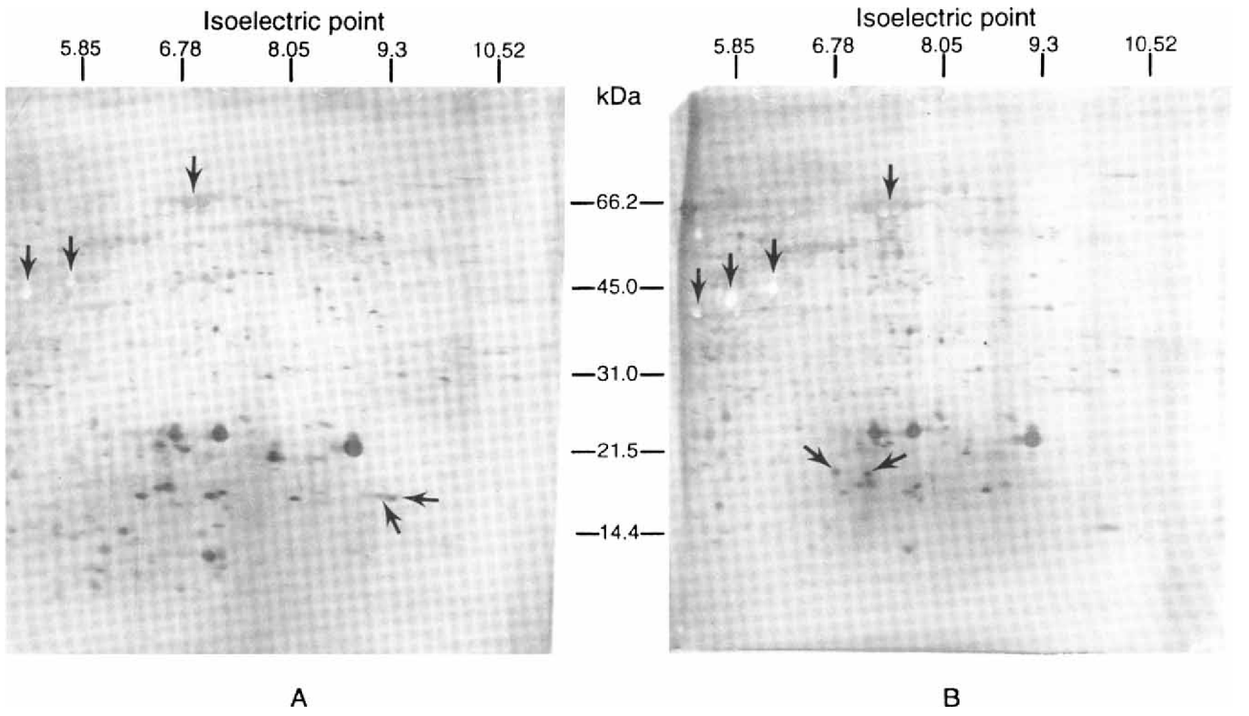



**Fig. 1. Two-dimensional gels comparing the peanut seed microsomal peptides of the high (A) and normal (B) oleic acid peanut isolines at the first developmental stage. Note the differences between the two peanut lines indicated by the arrows. A 18 kDa pair of polypeptides isoelectric focusing at about pH 9.3 is present in the high oleic acid line, while those polypeptides were not found in the normal oleic line, where instead, a pair of 20 kDa polypeptides focusing at pH 6.8 and 7.3 was found.**

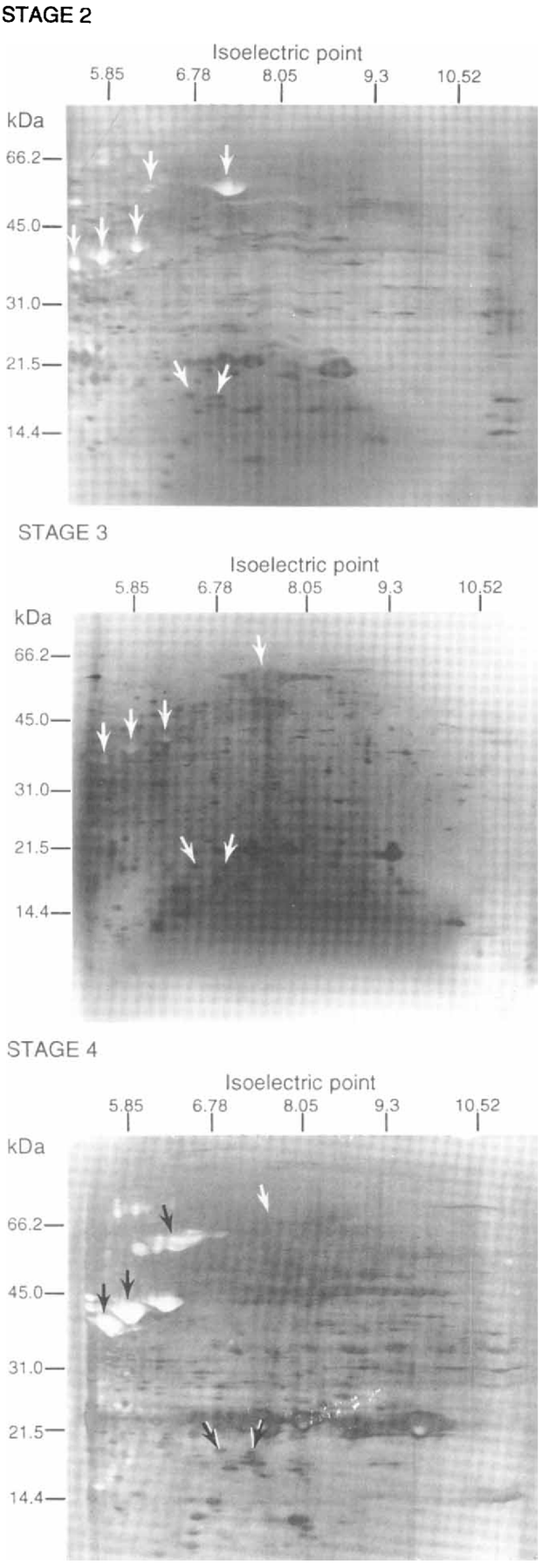
that they are very closely related, supporting the concept that the observed differing polypeptide pairs may be associated with the only observed phenotypic difference between the two lines, that being the difference in fatty acid desaturation. Only the earliest developmental stage of the high oleic acid isoline had the two 18 kDa polypeptides focusing at pH 9.3. The other developmental stages of either genotype did not have those polypeptides. This correlates with the reduced early production of linoleic acid in the high oleic acid peanut as compared to the normal peanuts (Powell *et al.*, 1990; D.A.K., unpublished). During developmental seed stages 2 through 4 the linoleic acid content, as a percent of total fatty acid, declined in a parallel fashion in both genotypes while the oleic acid percentage increased. Ray *et al.* (1993) reported that the $\Delta^{12}$-desaturase activity of immature (our stage 1 or earlier) high oleic acid peanuts was >10% that of the normal isoline, but they did not measure the desaturase activity of later maturity stages. The $\Delta^{12}$-desaturase complex is poorly understood, and so far has only been reported to function when intact and assembled on the microsomal membranes (Smith *et al.*, 1990; Ray *et al.*, 1993).

While we have only correlative evidence, we postulate that the 20 and 18 kDa polypeptide pairs could be components of the $\Delta^{12}$-desaturase complex, with the 18 kDa polypeptide pair being variant, less functional with lower desaturase activity, and producing less linoleic acid, thus increasing the oleic acid content. Since the 18 kDa polypeptides focusing at pH 9.3 are not found at later stages, they probably are under developmental control. It is interesting to note that the polypeptides differing between the genotypes occur in pairs.

Differences in seed polypeptide composition were evident when profiles from the different developmental stages were compared. Predominant polypeptide species, especially those that showed on the gels as unstained (cleared) areas, tended to increase in quantity with increasing seed maturity. This was true for both isolines, so only gels of the high oleic acid line are shown in Fig. 2. Subsequent analyses and re-staining of the silver stained gels with the much less sensitive Coomassie Blue stain indicated that the cleared areas in the silver stained gels contained high polypeptide concentrations, sufficiently high to inhibit the silver staining. Even in stage 1, the polypeptides in the unstained spots, (indicated by the vertical arrows in the upper left region of the gels) and the two poorly isoelectric focused polypeptides of about 66 kDa (indicated by a single vertical arrow just left of center) make up over half of the total protein on the gel. In stage 1, those cleared spots are most easily visualized in the normal oleic acid gel. The high concentrations of polypeptides in the cleared areas are most likely from protein bodies which co-sediment with microsomes (Harley and Beavers, 1989). That possibility is supported by the pattern similarity of the nonstaining spots to the major peanut seed storage protein patterns reported by Basha (1989).

## Literature Cited

1. Anderson, N. G. and N. L. Anderson. 1979. Molecular anatomy. Behring Inst. Mitt. 63:169-210.




**(Left)**

**Figure 2. Two-dimensional gel of peanut seed microsomal peptides from developmental stages 2, 3 and 4 of high oleic acid line F435. Note the differences in polypeptide patterns between the different maturity stages, especially the unstained areas marked by the arrows in the upper left quadrant of the gel and the two poorly isoelectric focused 66 kDa polypeptides just left of center. The unstained areas are due to polypeptide concentrations sufficiently high to inhibit silver staining.**


2. Basha, S. M. 1989. Accumulation pattern of arachin in maturing peanut seed. Peanut Sci. 16:70-73.
3. Grundy, S. M. 1986. Comparison of monounsaturated fatty acids and carbohydrates for lowering plasma cholesterol. New England. J. Med. 314:745-748.
4. Harley, S. M. and L. Beavers. 1989. Coated vesicles are involved in the transport of proteins during seed development in *Pisum sativum* L. Plant Physiol. 91:674-678.
5. Hurkman, W. J. and C. K. Tanaka. 1986. Solubilization of plant membrane proteins for analysis by two-dimensional gel electrophoresis. Plant Physiol. 81:802-806.
6. Knauft, D. A., K. M. Moore, and D. W. Gorbet. 1993. Further studies on the inheritance of fatty acid composition in peanut. Peanut Sci. 20:74-76.
7. McKeon, T. A. and P.K. Stumpf. 1982. Purification and characterization of the stearoyl-acyl carrier protein desaturase and acyl-acyl carrier protein thioesterase from maturing seeds of safflower. J. Biol. Chem. 257:12141-12147.
8. Moore, K. M. and D.A. Knauft. 1989. The inheritance of high oleic acid in peanut. J. Hered. 80:252-253.
9. Norden, A. J., D.W. Gorbet, D.A. Knauft, and C.T. Young. 1987. Variability in oil quality among peanut genotypes in the Florida breeding program. Peanut Sci. 14:7-11.
10. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250:4007-4021.
11. Pattee, H. E., E. B. Johns, J. A. Singleton, and T. H. Sanders. 1974. Composition changes of peanut fruit parts during maturation. Peanut Sci. 1:57-62.
12. Powell, G., A. Abbott, D. Knauft, R. Smith, and J. Barth. 1990. Oil desaturation in developing peanut seeds: studies of lipid desaturation in a peanut mutant that accumulates high levels of oleic acid. pp. 131-133. *In* Plant Lipid Biochemistry, Structure and Utilization. P.J. Quinn and J.L. Harwood, *(eds.)*, Proceedings of the Ninth Int'l. Symp. Plant Lipids, Wye College, Kent, UK.
13. Ray, T. K., S. P. Holly, D. A. Knauft, A. G. Abbott, and G. L. Powell. 1993. The primary defect in developing seed from the high oleate variety of peanut (*Arachis hypogaea* L.) is the absence of $\Delta^{12}$-desaturase activity. Plant Sci. 91:15-21.
14. Slack, C. R., P. G. Roughan, and J. Browse. 1979. Evidence for an oleoyl phosphatidylcholine desaturase in microsomal preparations from cotyledons of safflower (*Carthamus tinctorius* L.) seed. Biochem. J. 179:649-656.
15. Smith, M. A., A. R. Cross, O. T. G. Jones, W. T. Griffiths, S. Stymne, and K. Stobart. 1990. Electron-transport components of the 1-acyl-2-oleoyl-*sn*-glycero-3-phosphocholine Δ12-desaturase (Δ12-desaturase) in microsomal preparations from developing safflower (*Carthamus tinctorius* L.) cotyledons. Biochem. J. 272:23-29.
16. Stymne, S., A. K. Stobart, and G. Glad. 1983. The role of the acyl-CoA pool in the synthesis of polyunsaturated 18-carbon fatty acids and triacylglycerol production in the microsomes of developing safflower seeds. Biochim. Biophys. Acta 752: 198-208.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