Gas Chromatographic Analysis of Amino Acids in Oilseed Meals

Edith J. Conkerton

The amino acid contents of a soybean and a peanut meal were determined by the classical ion-exchange procedure and by gas chromatography. After derivatizing the amino acids as their *N*-trifluoroacetyl *n*-butyl esters, gas chromatographic analysis yielded reproducible quantitative data for all of the common protein amino acids except tyrosine, arginine, and histidine. Statistical analysis of the data indicated that gas chromatographic analysis gives results comparable to those obtained by ion-exchange chromatography. Some advantages and disadvantages of the gas chromatographic method are discussed.

The significance of amino acids as fundamental factors in the utilization of proteins was recognized in the early 1900's (Osborne and Mendel, 1914). Studies were limited, however, by the large amounts of starting material and the time required for an analysis. Not until the middle of the 20th century and the development of ion-exchange chromatography were protein and food chemists provided with "an exceedingly potent analytical tool" (Tristram and Smith, 1963). With automation of the ion-exchange procedure by Spackman *et al.* in 1958, the ultimate in speed and accuracy seemed to have been achieved. As time passed, however, analysts became dissatisfied with a 24-hr analysis time for a 2–3-mg sample, so instrumentation was improved. Current methods require less than 2 hr and less than 0.5 mg of sample. These improvements, however, increased purchasing and maintenance costs of this single-purpose unit—the automatic amino acid analyzer.

During this period other investigators studied amino acids with a different technique—gas chromatography. Minute quantities of food constituents, such as carbohydrates and fatty acids, were converted easily to volatile derivatives detectable by gas chromatography. However, because amino acids do not constitute a homologous series, it was difficult to find a derivatization procedure applicable to all. Preliminary work on the diverse procedures studied has been reviewed by Blau (1968) and Coulter and Hann (1971). Of the many derivatives investigated, those forming acyl esters seemed to offer the most promise. By 1968, Gehrke and coworkers had a quantitative procedure applicable to all the protein amino acids, based on the formation of their *N*-trifluoroacetyl *n*-butyl esters. This method retained the accuracy of the ion-exchange technique but lowered equipment cost and increased the speed and sensitivity of analysis. Except for data on a soybean protein reported by Gehrke *et al.* (1968), use of the method to analyze oilseed meals has not been reported. To establish the validity of amino acid data for oilseed meals obtained by gas chromatographic analysis, samples of peanut and soybean meals were analyzed by gas and ion-exchange chromatography. This paper compares data from the two methods and discusses some advantages and disadvantages of the gas chromatographic technique.

EXPERIMENTAL SECTION

Samples. For peanut meal dehulled intact Va-56 R peanuts were homogenized in 1:1 hexane–acetone in a Sorvall Omnimixer for 5 min at 5°. The homogenate was filtered under vacuum and then air dried to remove final traces of solvent. Soybean meal, supplied by J. F. Cavins, was representative of a sample used in a collaborative study of amino acid analysis (Cavins *et al.*, 1972).

Hydrolysis. All peanut meals and the soybean meals analyzed by gas chromatography were hydrolyzed in 6 *N* HCl by the procedure of Kurtzman *et al.* (1965) or Conkerton (1973).

Ion-Exchange Chromatography (IEC). Peanut meals were analyzed by the Moore and Stein (1963) procedure through a Technicon Auto-Analyzer. Soybean meals were not analyzed by IEC at this laboratory; the data reported are from Cavins *et al.* (1972).

Gas Chromatography (Gc). *Apparatus and Equipment.* Teflon-lined, screw-capped culture tubes containing Teflon-coated magnetic fleas were used for derivatization of the amino acids. To heat the samples, an aluminum block having holes to accommodate the culture tubes was mounted on a hot-plate magnetic stirrer. Temperatures were controlled to ±0.5°.

The gas chromatograph was a Packard Series 7300 having two flame ionization detectors, two electrometers, a linear temperature programmer, and two recorders. The stainless steel leads into the detector were replaced with narrow bore Teflon tubing. Ultra Pure hydrogen was prepared as needed with an Elhygen generator. Nitrogen, the carrier gas, and the compressed air were purified through filters of silica gel and molecular sieve, 200–300 mesh. Data were quantitated by connecting the electrometer output to an Infotronics CRS 12 AB digital integrator.

Reagents. All solvents were nanograde quality. They were not redistilled before use but, after opening, were protected from moisture by storage over nonindicating Drierite. *n*-Butyl alcohol was made 3 *M* with respect to HCl by bubbling the correct weight of HCl from a compressed gas cylinder through separate glass wool and sulfuric acid traps into a weighed amount of butanol. The Beckman amino acid calibration mixture, type I, was used routinely as the standard mixture. Amino acids, other than those in the standard mixture, were chromatographically pure from Schwarz/Mann, Orangeburg, N.Y. 4-(Aminomethyl)cyclohexanecarboxylic acid from Aldrich Chemical Co., Milwaukee, Wis., was used as internal standard. Trifluoroacetic anhydride (TFAA), 99+% pure, from Pierce Chemical Co., Rockford, Ill., was used as received but stored at 4° in a dessicator after opening. Column packings were either prepared as directed by Gehrke *et al.* (1968) or purchased from Supelco, Inc., Bellefonte, Pa., in Guaranteed Performance packings.

Derivatization of the Amino Acids. The following steps were followed.

(1) An appropriate aliquot of the amino acid calibration mixture or the hydrolysate (1–10 mg of protein) was transferred to the culture tube. (2) An exact amount of the internal standard (I.S.) was added. (3) Under a stream of nitrogen at 100°, with continuous stirring, the mixture was evaporated to dryness. (4) The mixture was covered and cooled to room temperature. (5) Residual moisture was

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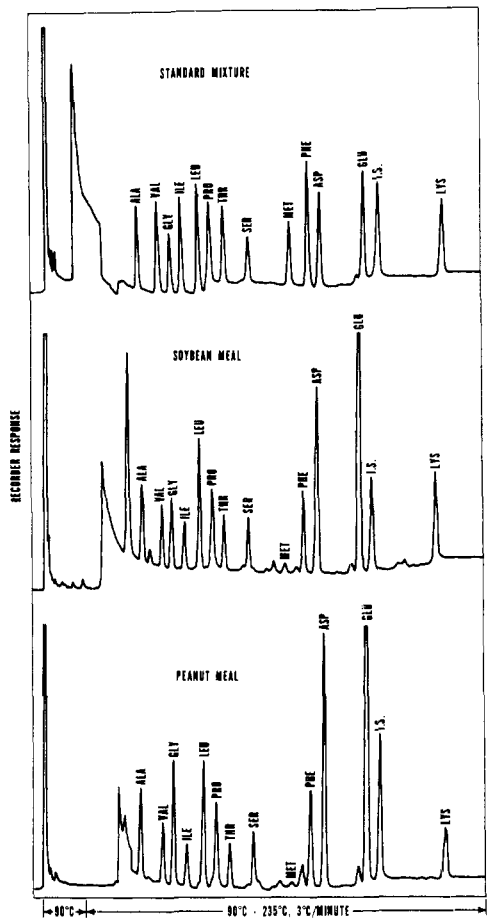
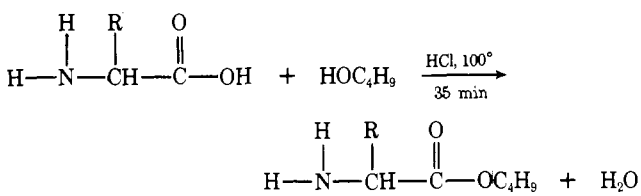
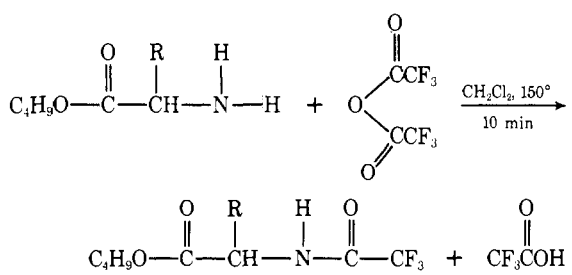


Figure 1. Typical separation of amino acid *N*-TFA *n*-butyl esters; column, 0.65 w/w % EGA on acid washed Chromosorb W, 1.5 m × 4 mm i.d., U-shaped glass.

removed by twice suspending the dried materials in methylene chloride and repeating steps 3 and 4. (6) An excess (1–3 ml) of butanol, 3 *M* with respect to HCl, was added and the tube capped tightly. (7) The mixture was esterified 35 min at 100°, stirring constantly



(8) The mixture was cooled to room temperature and then the solvent removed by repeating steps 3 and 4. (9) An excess of TFAA in methylene chloride (1:3, v/v) was added and the tube capped tightly. (10) The mixture was acylated for 10 min at 150°, stirring constantly



(11) The mixture was cooled to room temperature and transferred to a glass-stoppered vial. (12) Excess solvent and TFA were removed under a stream of N_2 at room temperature (to prevent losses in threonine and serine do not take to dryness). (13) The mixture was analyzed immediately or stored at 0° in a dessicator under nitrogen. Derivatives are stable up to 72 hr after derivatization. With slight modification, this is the semi-micro procedure described by Gehrke and Roach (1969).

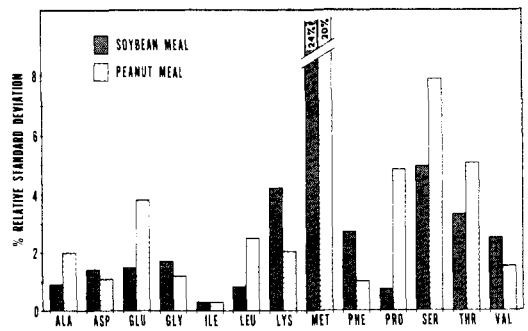


Figure 2. Relative standard deviation in amino acid determinations; variation between aliquots of one hydrolysate, gas chromatographic analysis.

Gas Chromatographic Conditions. Derivatives were separated on two 1.5 m × 4 mm i.d. U-shaped glass columns used in parallel. One, 0.65 w/w % ethylene glycol adipate on acid-washed Chromosorb W (EGA), separated most of the protein amino acids with temperature programming from 90 to 235° at 3°/min after a 5-min initial hold (see Figure 1). The second 1.0 w/w % OV-17 plus 2.0 w/w % OV-210 on Supelcoport (OV) separated the remaining acids, arginine, histidine, and cystine, with temperature programming from 100 to 235° at 3°/min. Flow rates employed were carrier N_2, 40 ml/min; H_2, 20 ml/min; and air, 400 ml/min.

Calculation of Data. The amino acid composition of the meals was calculated by the weight response method (Gehrke *et al.*, 1968).

RESULTS AND DISCUSSION

Tracings from the EGA column typical of a standard mixture of amino acids and a peanut and soybean meal hydrolysate are shown in Figure 1. The high glutamic and aspartic contents are characteristic of plant proteins. Several unidentified peaks are present in both meals, but all appear to be minor components or hydrolytic breakdown products or to have retention times not associated with known amino acid derivatives. A similar set of tracings from the OV column is not included because reproducibility from one batch of packing material to another was poor; separation of tyrosine and glutamic acid derivatives in equimolar mixtures of amino acids was possible, but the extremely high Glu/Tyr ratio in the meals caused a blending of the peaks making quantitation impossible.

Recently, Kwolek and Cavins (1971) reviewed the literature on amino acid determinations in plant seeds or seed products to establish precision levels in amino acid analyses of these materials. Using relative standard deviation (RSD) ("Guide for Measures of Precision and Accuracy," 1970) as a measure of precision with a 95% confidence limit, they concluded that amino acid values based on aliquots of the same hydrolysate should agree within 8% and values for independently hydrolyzed samples within 25% except for cystine, tryptophan, tyrosine, and methionine which may be higher. With this information as a guide, multiple samples of a soybean and of a peanut meal were analyzed for amino acid content and RSD's for each amino acid were determined.

Variations between aliquots of the same hydrolysate analyzed by gas chromatography are illustrated in Figure 2. Except for methionine, all RSD's were below 8%. High RSD values for methionine may be due to its instability coupled with its extremely low concentration (less than 1%) in both meals. The only other amino acids having

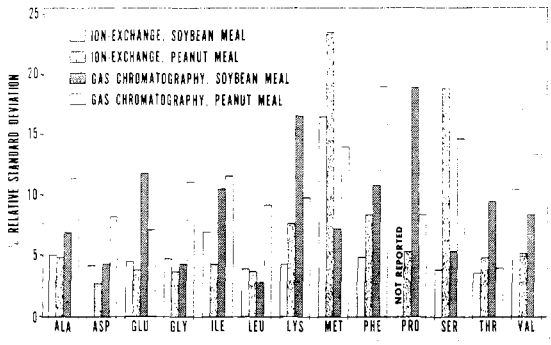


Figure 3. Relative standard deviation in amino acid determinations; variation between independently hydrolyzed samples, ion-exchange (data on soybean meal from Cavins *et al.*, 1972), and gas chromatographic analyses.

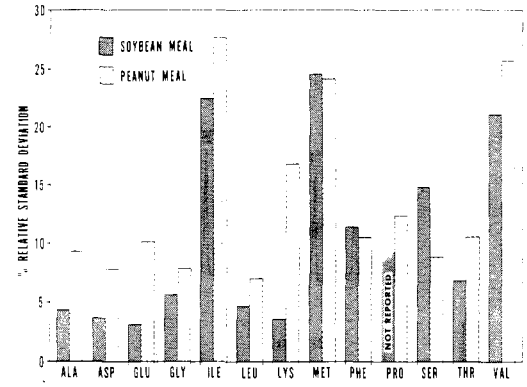


Figure 4. Relative standard deviation in amino acid determinations; variation between ion-exchange (data on soybean meal from Cavins *et al.*, 1972), and gas chromatographic methods.

RSD's above 3% for both meals are serine and threonine. The *N*-TFA *n*-butyl esters of these hydroxy amino acids are relatively unstable and subject to loss by evaporation. Replicate determinations of serine and threonine made within 24 hr of derivatization showed higher precision and values routinely decreased as time of storage increased. However, since the decreases were not consistent, all values were included in the determination of the reported RSD's.

Variations between independent hydrolysates of the soybean and peanut meals analyzed by ion-exchange and gas chromatography are illustrated in Figure 3. RSD's of all the amino acids are below the established maximum of 25%. In the soybean meal, RSD's of all amino acids are less than 11% except for methionine as determined by both methods and serine as determined by gas chromatography. In the peanut meal, RSD's of amino acids are not as consistent. The soybean meal may have been more uniform since it was a representative portion of a large commercial sample while the peanut meal was prepared in the laboratory from 1 kg of peanuts.

If it is assumed that independently hydrolyzed samples analyzed by one method have RSD's of 25% or less, variations between independently hydrolyzed samples analyzed by different methods should fall within that range provided the methods yield comparable results. In Figure 4, variations between ion-exchange and gas chromatographic methods of amino acid analysis show RSD's greater than 25% only for isoleucine and valine in the peanut meal. Even these are not exceedingly high, *i.e.* 27.5 and 26.7%, respectively.

Five known protein amino acids are absent from the data presented above—arginine, histidine, tyrosine, cystine, and tryptophan. The latter two were not included because they are adversely affected by acid hydrolysis—the only hydrolytic procedure used in this study. Determinations of arginine and histidine are the greatest obstacles to general acceptance of any gas chromatographic method for amino acid analysis. Gehrke *et al.* (1971) reported successful recoveries of the *N*-TFA *n*-butyl esters of arginine and histidine from the mixed OV column but this group of investigators has also reported problems in forming a single, stable derivative of each (Stalling and Gehrke, 1966; Gehrke *et al.*, 1969). In this laboratory several of the suggested modifications have been tried but reproducible peaks for histidine alone or in standard mixtures have not been obtained. Reproducible peaks were obtained for arginine alone but not in the calibration mixture. Investigators, other than those associated with Dr. Gehrke, have reported difficulty with both of these amino acids. Casagrande (1970) quantitated histidine values by calculation from the concentration of the combined aspartic acid-histidine peak on the OV column minus that of aspartic acid from the EGA column. The pictured response of arginine from the OV column was low, but Casagrande (1970) reported quantitative results. Bognar (1972), in reporting amino acid contents of meat, listed quantitative data for arginine from a nitrogen selective flame ionization detector but not from a regular flame ionization detector. Bognar did not list histidine for either detector.

Tyrosine should appear on the tracing from both columns. Although the response is low, similar to those of other hydroxy acids, it has been determined quantitatively (Gehrke *et al.*, 1971; Casagrande, 1970; Bognar, 1972). In this study tyrosine was derivatized and measured alone using the EGA column but not in the presence of a mixture of amino acids. As mentioned earlier the high Glu/Tyr ratio in peanut and soybean meals did not allow quantitation of tyrosine data from the OV column.

One advantage of the gas chromatographic technique over others is quantitation of data on nonprotein amino acids without increasing the time of analysis or requiring modification of the system. During this study, sarcosine, ornithine, citrulline, and *dl*-α-ϵ-diaminopimelic acid, added to the calibration mixture before derivatization, were separated quantitatively from the protein amino acids on the EGA column without modifying the chromatographic conditions. Using a similar technique, Raulin *et al.* (1972) quantitatively separated 18 nonprotein amino acids from the protein amino acids. Casagrande (1970) used the EGA column to obtain 30 distinct measurable peaks from a mixture of 33 amino acids. Overlapping occurred only for isoleucine and norvaline, glutamic acid and glutamine, and aspartic acid and asparagine. This technique, therefore, would be of particular advantage in a study of free amino acids in plant materials.

In the ion-exchange chromatographic method, each amino acid elutes from the column at a characteristic time but measurement of concentration depends on liberation of the α-amino group of the amino acid to form a colored ninhydrin complex, diketohydrindylidene-diketohydrindamine. In gas chromatography, a derivative of each amino acid is prepared, the derivatives are identified by retention times, and the area under the eluted peak is proportional to the concentration of the amino acid. Each peak could be identified positively by coupling the gas chromatograph with a mass spectrometer.

CONCLUSION

Data reported here indicate that the gas chromatographic method of analysis for amino acids is applicable to soybean and peanut meals. Reproducibility of quantitative analyses for 12 of the protein amino acids is equivalent to the classical ion-exchange procedure and data obtained by the two methods are comparable. Aside from the inherent advantages of gas chromatography, this technique offers distinct advantages over any other method of amino acid analysis in studies with plant materials containing both protein and nonprotein amino acids.

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Effect of Milling and Processing on the Selenium Content of Grains and Cereal Products

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The selenium content of grains and of the products and cereals derived from these grains was determined fluorometrically. Wheat flour and farina contained 14 and 29% less selenium, respectively, than the original raw wheat. Corn meal, flour, and grits contained 10–28% less selenium than did the raw corn. Milling of oats and rice caused little change in selenium concentration of those grain fractions destined for consumer use. Little or no decline in selenium concentration occurred during the manufacture of nonsugared corn or wheat breakfast cereals. There was a significant decrease in the concentration of selenium in sugared corn and sugared wheat breakfast cereals which was apparently due to dilution of selenium by the sugar. The decrease in selenium concentration in consumer products due to milling or processing of grains appeared to be less than that reported with several other nutritionally essential trace elements.

Selenium poisoning of farm animals due to the consumption of seleniferous plants grown on soils containing high levels of the element has long been recognized as a practical agricultural problem in certain regions of the United States (Moxon and Rhian, 1943; Rosenfeld and Beath, 1964). Because of the toxicological significance of selenium, analytical surveys were carried out to ascertain the selenium content of feedstuffs grown in seleniferous zones (Lakin and Byers, 1941; Smith and Westfall, 1937). Later research, however, has shown that the element can also have beneficial nutritional effects when present in the diet in trace amounts (Schwarz and Foltz, 1957), and selenium deficiency is now also considered a practical agricultural problem in some areas of the United States and some other parts of the world (Kubota *et al.*, 1967; Muth *et al.*, 1967). Although no selenium requirement has been established for humans, considerable experimental evidence indicates that the levels of 0.1 ppm of selenium in the feed for livestock and 0.2 ppm in the feed for turkeys will prevent deficiencies (Oldfield *et al.*, 1971). Because of the newly discovered nutritional importance of selenium, analytical surveys have been performed on foods and feedstuffs from nonseleniferous areas to determine whether or not dietaries contain adequate amounts of this essential trace element (Arthur, 1972; Morris and Levander, 1970; Oelschlager and Menke, 1969; Suchkov, 1971).

Grains, grain products, and cereals are important components of most ordinary diets, but the processing of grains can result in lower concentration of many nutritionally desirable trace elements in the flour (Czerniejewski *et al.*, 1964; Schroeder, 1971). The purpose of this work was to determine whether the processing of grains resulted in any decrease in selenium.

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EXPERIMENTAL SECTION

Description of Samples. The samples of original raw grains and grain products were collected by the millers on the same day of the milling operation so that any day-to-day variation in the selenium concentration of the samples obtained would be minimized. A balance flow sheet which showed the yield of each product obtained in any given process batch was also provided by the manufacturers. If the sample was not already in a finely ground state as received, a 20-g subsample was ground in a Wiley mill and mixed thoroughly. All samples were stored at −6° a 10–30% decrease in the selenium concentration of the consumer product. This finding is in agreement with a