(17) Phillips, W. R., MacQueen, K. F., Poapst, P. A., *Proc. Intern. Congr. Refrig. 10th* **3,** 176 (1960).
(18) Pratt, G. B., Ecklund, O. F., *Food Technol.* **10,** 496 (1956).
(19) Romani, R. J., van Kooy, J., Robinson, B. J., *Food Irradiation* **2,** 11 (1961).
(20) Salunkhe, D. H., *Econ. Bot.* **15,** 28 (1961).
(21) Saravacos, G. D., Halzipetrou, L. R., Georgiadou, E., *Food Irradiation* **3,** 6 (1962).
(22) Smock, R. M., *Cornell Univ. Agr. Expt. Sta. Bull.* **750,** 1948.
(23) Smock, R. M., Sparrow, A. H., *Proc. Am. Soc. Hort. Sci.* **70,** 67 (1957).
(24) Sparrow, A. H., Christensen, E., *Nucleonics* **12,** No. 8, p. 16 (1954).

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FOOD FLAVORS

Isolation and Localization of the Precursors of Roasted Peanut Flavor

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This work represents initial attempts to isolate and identify precursors of roasted peanut flavor. The techniques used for these studies revealed useful information concerning the locale and molecular origin of flavor precursors within peanut cotyledons. Flavor does not appear to arise from the large globular proteins nor from carbohydrate material per se. Flavor does arise from one or a combination of ninhydrin-positive compounds and basic compounds. Apparently, flavor originates from rather specific types of micromolecules rather than from general, macromolecular, cellular components such as the large globulin proteins and the starches.

Very little is known about the compounds responsible for the typical flavor of roasted peanuts or about the precursors which give rise to flavor during roasting. At the time of a review by Hoffpauir (*4*), a few of the components of gases given off during roasting had been identified, and a measurable decrease in total sugars during roasting had been noted (*6*). Gaseous components identified included derivatives of tetrahydrofuran, ammonia, hydrogen sulfide, and diacetyl. The major gaseous component was carbon dioxide. Also, Pickett and Holley (*6*) had shown that mixtures of amino acids and carbohydrates reacted to produce tetrahydrofuran derivatives along with noticeable browning and discernable aromas. On the basis of the meager information available, Hoffpauir speculated on the precursors responsible for the formation of peanut flavor during roasting. The two main protein components of cotyledons, arachin and conarachin, which have been classified as reserve proteins (*7*), were implicated as precursors on the basis of their unusually high sulfur content. The implication was made to explain the presence of sulfide in roasting gases. Sucrose was implicated as a precursor on the basis of the loss of total sugars during roasting and on the basis of the appearance of tetrahydrofuran derivatives. Peanut cotyledons contain about 4.5% total disaccharides of which sucrose is the major component. Protein-bound and free amino acids were implicated for similar reasons. Since 1953, nothing in the literature had added significantly to this sketchy picture.

Work reported here represents initial results of an integrated program to improve peanut quality by identifying the precursors which give rise to typical peanut flavor during roasting.

Reagents

Optical hexane was prepared by distilling high purity *n*-hexane over KOH pellets onto a silica gel column. The hexane collected from the column lacked absorption in the 230- to 260-mμ range compared to air.

Sephadex gels, obtained from Pharmacia, Uppsala, Sweden, were swollen, washed, and packed on columns according to the procedures outlined by Fasold, Grundlach, and Turba (3).

Refined cottonseed oil was obtained from the Great Western Foods Company, Fort Worth, Texas.

Carbon tetrachloride, reagent grade, was used for adjustment of the specific gravity of the cottonseed oil.

Spray reagents used for detection of spots on paper chromatograms were: 0.2% ninhydrin in water-saturated *n*-butanol; equal volumes of 0.1N silver nitrate and 5N ammonium hydroxide; and 0.4% bromocresol green in 95% ethanol. Chromatograms were developed on Whatman No. 1 filter paper in *n*-butanol–acetic acid–water (8:2:2) solvent.

Procedures

Roasting Studies. Roasting data were obtained by placing 38 grams of raw peanuts in the wire basket of an electrically heated rotisserie (General Electric) preheated to the desired temperature. Heating was continued until roasting was complete as judged by color and taste. If an under- or overroast was indicated, the process was repeated until a satisfactory roast was obtained. In one case, peanuts of Starr variety were roasted by placing them in the roaster at room temperature and increasing the temperature to the desired level as rapidly as possible. Logarithms of the time necessary for complete roasting of three varieties of Spanish peanuts, Starr, Spantex, and Argentine, were plotted versus the inverse of temperatures used.

Roasting Individual Fractions from Fractionation Studies. Individual frac-

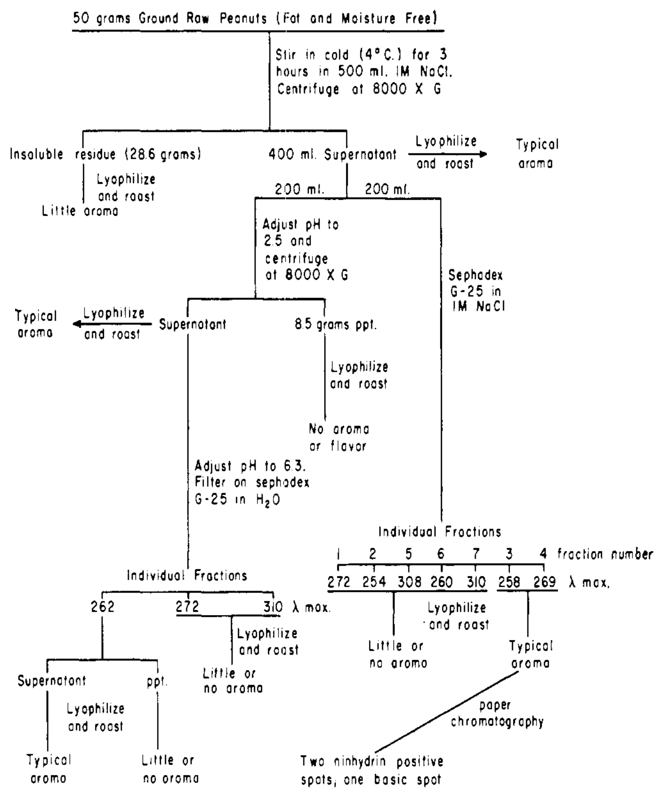

Figure 1. Flow chart showing procedure for separation of flavor precursors from mature peanut cotyledons by using gel filtration techniques

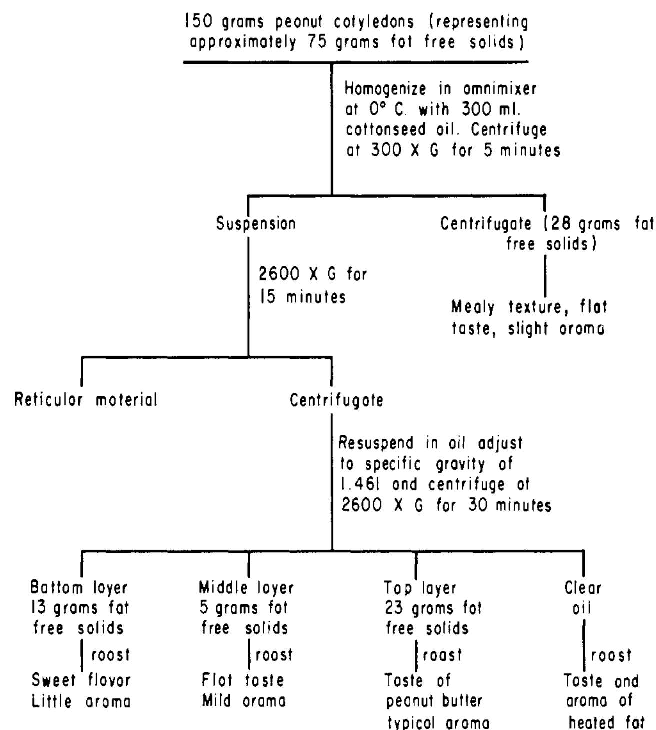

Figure 3. Flow diagram showing procedure for fractionation of particulates from mature peanut cotyledons

tions obtained from the gel filtration and particulate fractionation experiments were macerated with solvent-extracted peanut oil, wrapped in aluminum foil, and roasted at 400° F. in the rotisserie. Since the samples could not be observed, several roastings were performed on each fraction before a satisfactory roast was obtained, as judged by color and aroma, and taste where possible.

Precursor Isolations by Gel Filtration. The flow diagram in Figure 1 briefly outlines the procedure for separation of flavor precursors by this technique. Fifty grams of the dry, fat-free, finely ground cotyledons were extracted with 500 ml. of 1*M* NaCl for 3 hours at 4° C. Suspension was maintained by gentle stirring on a magnetic stirrer insulated from the beaker by an asbestos pad. Next, the suspension was filtered through a plug of glass wool, the filtrate centrifuged at 8000 × G for 30 minutes at 4° C., and the supernatant liquid decanted. Two-hundred milliliters of this solution were placed on a 2-liter Sephadex G-25 column in 1*M* NaCl and developed with 1*M* NaCl at 100 to 200 ml. per hour. Aliquots of each 12-ml. fraction collected were diluted when necessary and scanned on a Cary Model 14 recording spectrophotometer to observe the magnitude of peak absorbances and shifts in wavelength of maximum absorbances that occurred when the composition of absorbing material in the eluant changed. In this way, absorbance

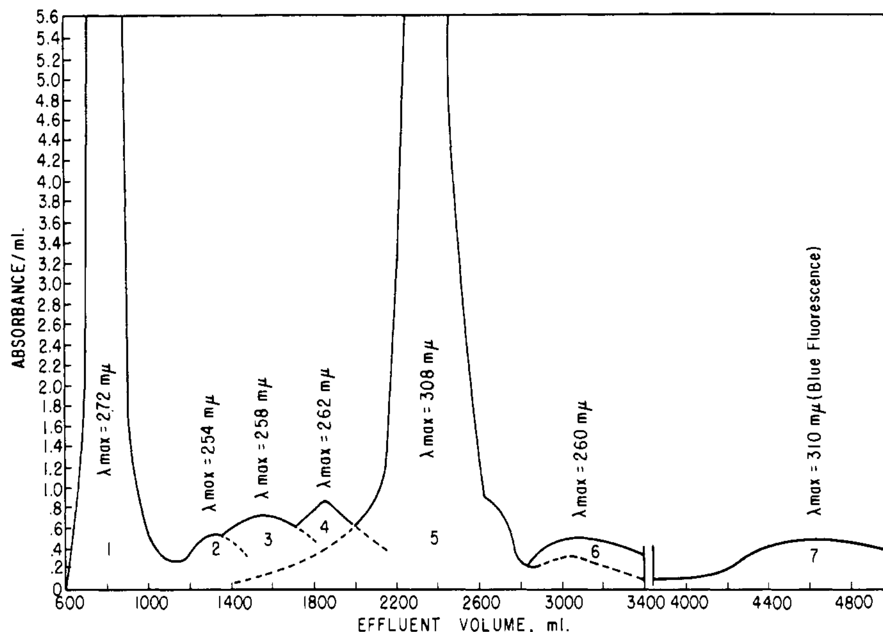

Figure 2. Elution pattern of saline extract of peanut cotyledons from Sephadex G-25 column in 1M NaCl having the following characteristics: total volume (V_t), 2010 ml.; void volume (V_0), 650 ml.; internal volume (V_i), 960 ml.; and flowrate, 200 ml. per hour

per milliliter at maximum absorbances of the eluate was calculated and plotted versus volume of eluant to provide an elution pattern. This is shown in Figure 2 along with the wavelength of maximum absorbance of each peak. Contents of tubes lying between the minima on the curves of the elution pattern were combined, assigned fraction numbers (Figure 2), lyophilized, and the resultant solid-salt mixtures roasted. Aliquots were taken from alternate tubes in each fraction for paper chromatography before contents of the tubes were combined.

An alternate fractionation procedure

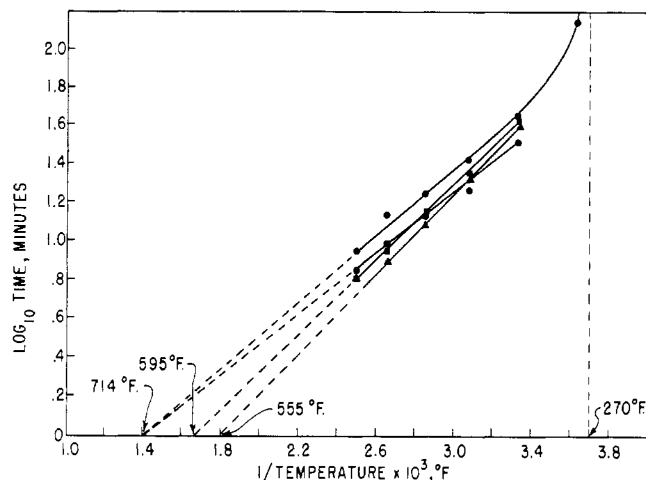

Figure 4. Roasting studies on three peanut varieties: Plot of the logarithm of the time necessary to roast 28 grams of mature peanuts versus the inverse of the temperature used

■ Argentine; ▲ Spanette; ● Starr

used involved adjusting the saline solution obtained after centrifugation to pH 2.5 by using 2N HCl. The resulting heavy white precipitate was centrifuged at 8000 × G after standing at 4° C. overnight. The supernatant was adjusted to pH 6.3 and filtered using 1M NaCl on a 2-liter column of Sephadex G-25 in 1M NaCl or alternatively on the same column recycled into the water form using water as the eluant, for separation of components which did not precipitate at pH 2.5.

Precursor Localization by Particulate Fractionation. Figure 3 contains a flow chart outlining the major steps involved in this nonaqueous, density gradient centrifugation procedure. With the variety of peanuts used in the author's laboratory, the density gradient centrifugation procedure outlined by Dieckert *et al.* (2) resulted in the aleurone grains, protein bodies, and starch grains sedimenting in the same fraction. Consequently, specific gravity adjustments other than those used by Dieckert were made in an attempt to effect a similar separation.

Peanut cotyledons were prepared in the same manner as for the gel filtration experiments. One hundred and fifty grams of the cotyledons were homogenized in an Omnimixer (Serval) fitted with a 400-ml., stainless steel cup for 2 minutes in 300 ml. of oil. This was done in three separate batches, 50 grams at a time with 100 ml. of cottonseed oil. The thick brei were combined and centrifuged at 300 × G for 5 minutes. The supernatant was then decanted; the centrifugate was resuspended in 300 ml. of oil and recentrifuged at 300 × G for 2 minutes. Microscopic examinations indicated the combined centrifugate was mostly vascular tissue with some particulate material remaining. The supernatants were combined and centrifuged at 2600 × G for 15 minutes, leaving most of the reticular material, in suspension. At this point, the centrifugate was composed mostly of the three particulate bodies—aleurone grains, protein bodies, and starch grains. Some needle-like bodies were also present. When the centrifugate was resuspended in oil, adjusted on a Westphal balance to a specific gravity of 1.461 with carbon tetrachloride (22° C.), and centrifuged at 2600 × G for 30 minutes, three centrifugates and a clear oil medium resulted. The layer floating on the oil (top layer) was removed with a spatula and the oil decanted. A second layer adhering to the side of the bottle (middle layer) was next removed with the aid of a large rubber policeman, and, finally, the bottom layer was removed in a similar manner.

All the fractions except the reticular suspension were washed free of oil on filter paper with carbon tetrachloride, dried at 40° C., and stored in a freezer. Each fraction was suspended in oil, examined under the microscope, heated to 180° C. for periods of 5, 10, and 15 minutes, and re-examined. Also, these fractions were roasted in the rotisserie in the usual manner for taste, color, and aroma observations.

Results and Discussion

Significance of Roasting Studies. Considering what has been learned of the morphology of the parenchymal cells of cotyledons (7), these cells represent peculiar reaction vessels. The fact that they are highly compartmentalized, relatively nonfluid reaction media led the authors to predict that the flavor precursors in these cells must also be compartmentalized and extremely restricted in motion so that the probability of two separate molecules colliding during the heating process would be very low. If this prediction were true, flavor formation would be essentially an intramolecular conversion rather than intermolecular.

Data from roasting studies presented in Figure 4 established some points with respect to the nature of the reactions leading to roasted peanut flavor and were indicative of others. The critical minimum temperature for flavor formation is close to 270° F. (132° C.) since the upper curve in Figure 4 approaches asymptotically a line perpendicular to a $1/T$ value corresponding to 270° F. Several attempts to roast the three varieties of peanuts indicated in Figure 4 below this temperature failed. This is slightly higher than the minimum temperature of 120° C. reported by Pickett and Holley (*6*), but a different variety of peanuts was used in their experiments. Seemingly, temperatures of this order exclude the possibility of enzymatically catalyzed reactions giving rise to flavor components. That flavor formation (precursor degradation) is a pyrolysis reaction dependent solely on energy supply seems to be established by the fact that in the range of temperatures studied in which roasting readily occurred, 300° to 425° F., the semilogarithmic plots of time versus $1/T$ were linear, obeying the Arrhenius concept of the effects of temperature on reaction rates.

In addition, the fact that linear plots were obtained and extrapolated portions of the two linear curves for the same variety (Starr) heated at different rates intersected, led the authors to conclude that flavor formation resulted from intramolecular pyrolyses. This conclusion was based on the assumption that the compartmentalized and nonfluid state of mature peanut cotyledons would greatly inhibit intermolecular processes. The possibility exists that the oil droplets surrounding the aleurone grains (*7*) could supply the fluid medium needed for intermolecular processes.

The answer to the question of intra- versus intermolecular processes is especially significant to the isolation of flavor precursors. If intramolecularity were the rule, one could pyrolyze precursor fractions and study their particular contribution to typical flavor without numerous recombinations of individual fractions. Each component would make its particular contribution to flavor without the presence of other components. In the succeeding discussion of precursor isolation, it is apparent that the authors did not attempt to make numerous recombinations of fractions; roasting of individual fractions was apparently sufficient to follow precursor isolations.

Isolation of Flavor Precursors by Gel Filtration. Because of the implication that proteins might serve as flavor precursors, a procedure was formulated

which would provide the separation of the two main globulins, arachin and conarachin (*1*, *5*), of cotyledon parenchymal cells as well as other cellular components. Saline solutions have been used to solubilize globulins, and Naismith and McDavid (*5*) successfully used *1M* salt solutions to extract peanut seed globulins for ultracentrifugal studies; thus, 1*M* solutions were used for this procedure. Sephadex G-75 was first chosen on the basis that arachin was large enough to be totally excluded from the gel, thus appearing at the eluting front. Conarachin would be close to the borderline of size for exclusion and would undergo some partitioning with the gel internal phase. Two very large peaks were observed in the elution patterns in the positions expected. One contained the largest amount of protein material on a nitrogen basis and appeared at the eluting front while the other appeared immediately behind it with a partition coefficient (K) equal to 0.6. Roasting results indicated that neither fraction contributed to typical peanut aroma or taste. Consequently, Sephadex G-25 was used for subsequent fractionations in which the two protein fractions appeared as a single peak at the eluting front (Figure 2). This choice allowed a cleaner separation of smaller components having K values greater than 0.

Salt extraction solubilized an average of 45% of the total solids including the flavor precursors. The insoluble residue from several extractions failed to contribute to typical peanut aroma or flavor when roasted. Components responsible for typical roasted peanut aroma were found in fractions 3 and 4 (Figure 2), which had maximum absorbances at 258 and 262 mμ, respectively. Other fractions did not contribute to flavor in any of the many separations made. Complete desalting of these two fractions was never achieved because these compounds have K values very near that of NaCl (K=1). Consequently, roasting studies were done on the dry salt-solid mixtures making any taste observations difficult to interpret even though aroma observations were easily obtained. However, a very marked browning consistently occurred only in the fractions which developed typical peanut aroma. This browning progressed into a deep chocolate color, as roasting continued, without developing a charred aroma. This marked color change was considered to be a property of flavor development and an excellent marker for following flavor precursor isolation.

Paper chromatograms revealed the presence of two ninhydrin-positive spots—one remaining near the origin, and one moving with an R_f of 0.10. A highly basic spot (R_f 0.19) which reacted yellow to ninhydrin was also present. Although the same three components were present in both fractions 2 and 3, most of the ninhydrin-positive material was in the peak absorbing at 258 mμ. The ammoniacal silver nitrate test for sugars was negative in all chromatograms.

When the alternate procedure involving pH adjustment before gel filtration was used, about 95% of the protein material in fraction 1 (λ_{max} = 272) was precipitated, and the only other absorbing materials remaining in solution were those absorbing at 258 to 262 mμ and at 310 mμ. Approximately 34% of the soluble material was precipitated leaving in solution about 32% of the weight of original fat-free solids extracted. This protein precipitate was 15.7% nitrogen by Kjeldahl analysis and was devoid of any aroma or flavor when roasted. On the other hand, the supernatant maintained typical aroma characteristics after drying and roasting. When this supernatant was filtered on a 2-liter Sephadex G-25 column in water, the material having an absorbance maximum at 262 mμ was eluted between 1296 and 1946 ml. as a suspension. The suspension was allowed to settle; the supernatants of all the tubes of this fraction were decanted and the precipitates combined and roasted. No typical aroma or taste was observed in the precipitate (absorbing material), but the combined supernatants did develop typical aroma. The profound tendency for browning was still present. This supernatant still contained some salt which made the taste observations difficult to interpret. Little or no aroma developed in the fractions containing material absorbing at 272 mμ and 310 mμ.

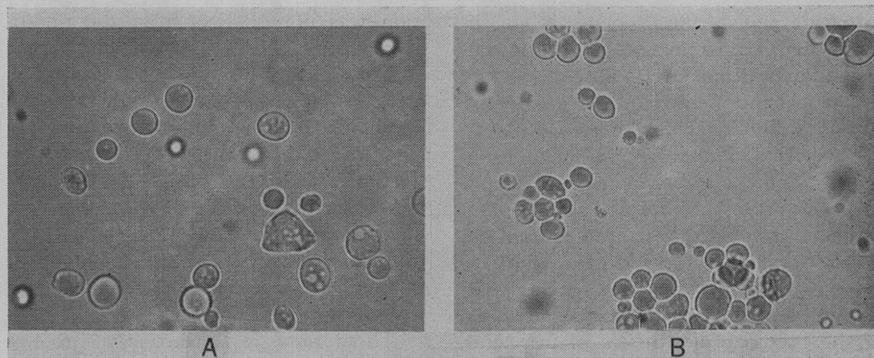

Figure 5. Photomicrographs of aleurone grains and protein bodies (top layer), magnification 1200×, isolated according to the prescribed procedure

(*A*) Before heating, suspended in peanut oil
(*B*) After heating 30 minutes at 180° C., suspended in oil, showing clumping

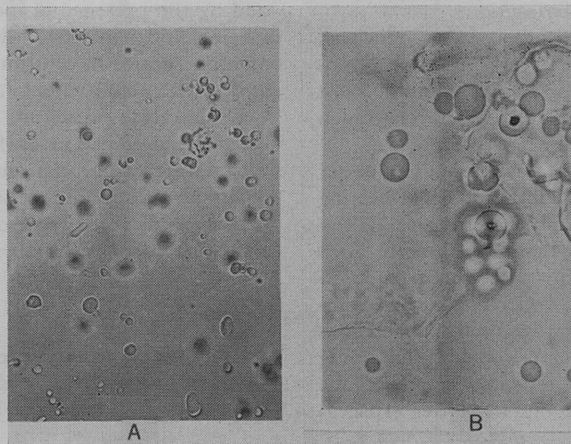

Figure 6. Photomicrographs of starch grains (bottom layer) isolated according to the prescribed procedure

(*A*) Before heating, suspended in oil, magnification of 520×
(*B*) After heating, suspended in oil, magnification of 1200×, showing donut-like holes in two of the starch grains

Localization of Flavor Precursor. The observation by Woodroof and Leahy (*7*) that oil pressed from peanuts contained aleurone grains, among other particulates, coupled with the observation in this laboratory that the settlings from oil pressed from freshly roasted peanuts were extremely potent carriers of peanut aroma, led to this study of the particulates. Examination of the three

layers for typical birefringence of starch grains under cross-polarized light showed that starch grains were absent in the upper layer, appeared only occasionally in the middle layer, and made up about 80% of the total bodies in the bottom layer. Some large protein bodies and aleurone grains as well as vascular and reticular materials were also present in the bottom layer (Figure 6). The distinction between aleurone grains and protein bodies was made on the basis of the presence or absence of inclusion bodies within the particulates. On this basis, there appeared to be little difference between the particulates of the upper and middle layers. Weights of particulates obtained from each of the three layers as well as the weight of vascular material are included in Figure 3. Although all fractions roasted produced some typical peanut aroma and taste, the upper layer (aleurone grains and protein bodies) was by far the most potent in aroma and tasted very much like peanut butter.

Examination of these fractions under the microscope before and after heating revealed that the protein bodies and aleurone grains maintained their gross structural integrity throughout the heating process (180° C.) which was comparable to normal roasting temperatures (Figure 5). Heating caused clumping of particulates and lightening of the inclusion bodies, but the latter observation was not obvious from the photomicrographs in Figure 5. The starch grains (lower fraction of Figure 6*A*) ruptured during heating producing small donut-like holes in the center of the grain (Figure 6*B*) and an effervescence in the medium, apparently due to the release of water vapor. This effervescence began at about 110° C. That starch grains were the only particulates which ruptured was shown by examining particulates for typical birefringence.

These data established the protein-body aleurone grain fraction as the specific location of flavor precursors and implicated the aleurone grains more strongly than the protein bodies. However, this choice cannot be made until a good separation of these two bodies is effected, as judged by chemical analysis and by the presence of inclusion bodies. Chemically, the two differ in that aleurone grains have a much higher ash and phytic acid content (*2*).

Using this procedure to obtain a fraction rich in precursors followed by gel filtration for separation and purification of individual components promises to provide sufficient precursor material for the elucidation of structures.

Acknowledgment

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Literature Cited

(1) Danielson, C. E., *Plant Physiol.* **7,** 215 (1956).
(2) Dieckert, J. W., Snowden, T. E., Jr., Moore, A. T., Heinzelman, D. C., Altschul, A. M., *J. Food Sci.* **27,** 321 (1962).
(3) Fasold, Hugo, Grundlach, Gerd, Turba, Friedrich, "Chromatography," Erich Heftmann, ed., p. 413, Reinhold, New York, 1961.
(4) Hoffpauir, C. L., J. AGR. FOOD CHEM. **1,** 668 (1953).
(5) Naismith, W. E. F., McDavid, H. M. P., *J. Appl. Chem.* **8,** 605 (1958).
(6) Pickett, T. A., Holley, K. T., *Georgia Agr. Expt. Sta. Tech. Bull.* **1** (1952).
(7) Woodroof, J. G., Leahy, J. F., *Georgia Agr. Expt Sta. Bull.* **205,** 1 (1940).

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COTTONSEED FLOUR

Free and Total Gossypol, Epsilon-Amino Lysine, and Biological Evaluation of Cottonseed Meals and Flours in Central America

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RECENTLY, Bressani *et al.* (*4–7*) have described the development of INCAP Vegetable Mixture 9, made of corn 28%, sorghum 28%, cottonseed flour 38%, torula yeast 3%, and dehydrated leaf meal 3%. This mixture, developed for the supplementary feeding of humans, had a high nutritive value (*15*). Its successful commercial production depends upon finding a cottonseed flour produced in Central America that is suitable for human consumption.

The specifications for human grade cottonseed flour, established in 1960 (*17*), required that it should contain not less than 50% protein, 3.6 grams of lysine per 16 grams of N, and not more than 0.055% of free gossypol and 1.00% of total gossypol. This paper reports on the differences in proximate composition, free ϵ-amino lysine, free and total gossypol, and nutritive value among cottonseed flours produced in the laboratory by screening commercial cottonseed meals.

Material and Methods

Five 400-pound samples of cottonseed meal, one from each of five mills located in Central America, were collected from the daily production and were brought to INCAP where they were stored at 4° C. until used. The samples were identified as follows: B, KH, AGSA, DS, and NC. Batches of 150 grams each from all the meals, each of which had different degrees of grinding, together with 10 small crystal balls, were placed on top of a series of screens (20, 40, 60, 80, 100 mesh) and shaken for 20 minutes in a laboratory shaker set at medium speed. After each run, the material which remained and that which had passed through the screens were weighed, and the percentage of each fraction was calculated. This process was continued until 1 kilogram of the lowest yielding fraction was obtained. Samples of the original meal and of each fraction were then analyzed for their proximate chemical composition by the A.O.A.C. official methods of analysis (*3*), and for their free and total gossypol, by the A.O.C.S. official methods (*2*). The free ϵ-NH_2 lysine content of each fraction was obtained by the method of Conkerton and Frampton (*9*).

For the biological trials, the original