Histochemical Localization of Amino Acids and Sugars in Peanut Cotyledons for Light Microscopy[1]

C.T. Young[2], H.E. Pattee[3]*, W.E. Schadel[4], and T.H. Sanders[5]

ABSTRACT

Light microscopy staining methods for amino acids and sugars were adapted for glutaraldehyde vapor-fixed, freeze dried, plastic embedded tissue of peanut cotyledons (*Arachis hypogaea* L.). These methods revealed the locale of amino acids and sugars, which are regarded as precursors of roasted peanut flavor compounds. Amino acids were localized in the protein bodies and cytoplasmic network by Coomassie Brilliant Blue G-250, Orange G, and diacetylbenzene. Sugars were localized in the protein bodies and cytoplasmic network by Alcian Blue - PAS procedure and an adaptation of the Okamoto method for sugars. Localization of the amino acids and sugars provide further evidence to the importance of the protein bodies in the cotyledon in the production of roasted peanut flavor.

Key Words: Histochemistry, light microscopy, roasted flavor precursors, Coomassie Brilliant Blue G-250, Orange G, diacetylbenzene, Alcian Blue - PAS, Okamoto method.

Roasted peanuts and their associated products are very appealing commodities with a desirable and unique flavor. Protein-bound and free amino acids in combination with sugars (sucrose, glucose, fructose, raffinose, stachyose) have been implicated as the primary precursors for the volatile flavor compounds of roasted peanuts (Picket and Holley, 1952; Mason and Waller, 1964; Mason *et al.*, 1966; Newell *et al.*, 1967; Vercellotti *et al.*, 1993). Certain sensory attributes that are important to overall roasted peanut flavor have been shown to be heritable traits (Pattee and Giesbrecht, 1990; Pattee *et al.*, 1993, 1995, 1998). Oupadissakoon and Young (1984) have modelled roasted peanut flavor and found that the best 10-variables model for predicting roasted peanut flavor used the concentration of eight different amino acids, sucrose, and total sugar from raw peanuts. Pattee *et al.* (2000) investigated possible relationships between heritable sensory traits and found a positive correlation of total sugars with sweetness, a positive correlation of total sugars with roasted peanut attribute, and negative correlation of total sugars with bitterness and astringency. The expected generalized relationship of total sugars or sucrose to sweetness could not be established because the relationship was not the same across all market-types.

Although a relationship between amino acids, sugars and roasted peanut flavor seems to be evident, there is little or no information about the physical relationships of these components within peanut cotyledon cells. The light microscopy methods reported in this study provide alternative histochemical means for localizing these amino acids and sugars in peanut cotyledons before roasting and understanding these relationships.

Materials and Methods

Mature peanuts seed (*Arachis hypogaea* L., cv. NC 7) were obtained from the North Carolina Peanut Growers Assoc. They were grown under standard cultural practices recommended for North Carolina.

Glutaraldehyde Vapor Fixation.

Whole peanut cotyledons were skinned and split into separate cotyledons before fixation. The split cotyledons were then placed in 10 mL beakers, and each beaker was placed in a specimen jar containing 2 mL of 70% glutaraldehyde. Each jar functioned as an individual vapor-fixation chamber for the cotyledons in the beakers after the lids of the specimen jars were sealed. Cotyledons were vapor-fixed for a period of 4 wk at 4 C.

Freeze Drying.

After glutaraldehyde vapor fixation, cotyledons were freeze dried for 16 hr before absolute ethanol dehydration to remove any residual moisture.

Absolute Ethanol Dehydration.

After glutaraldehyde vapor-fixation and freeze drying, tissue blocks (1 mm^3) were cut from outer and inner surfaces of all peanut cotyledons for dehydration in absolute ethanol (except for tissue blocks used in the adaptation of the Okamoto method - see procedure for these tissue blocks under the applicable stain method). Tissue blocks were dehydrated for 5 min intervals in two changes of absolute ethanol before infiltrating with Spurr's resin.

Embedding with Spurr's Resin.

Dehydrated tissue blocks were infiltrated and embedded with Spurr's resin using the methodology of Spurr (1969) for long pot-life resin.

Preparation for Light Microscopy.

Sections, 3 µm in thickness, were cut from embedded samples using a Reichert ultramicrotome and glass knives. After mounting sections on glass slides, the sections were

[1]The research reported in this publication was a cooperative effort of the Agric. Res. Serv. of the U. S. Department of Agriculture and the North Carolina Agriculture Research Service, Raleigh, NC 27695-7643. The use of trade names in this publication does not imply endorsement by the U. S. Department of Agriculture or the North Carolina Agriculture Research Service of the products named, nor criticism of similar ones not mentioned.

[2]Professor, Dept. of Food Science, North Carolina State Univ., Raleigh, NC 27695-7624. Deceased.

[3]Research Chemist, U. S. Department of Agriculture, Agricultural Research Service, Dept. of Botany, North Carolina State Univ., Raleigh, NC 27695-7625.

[4]Microscopist, Dept. of Food Science, North Carolina State Univ., Raleigh, NC 27695-7624.

[5]USDA, ARS, Market Quality and Handling Res., North Carolina State Univ., Box 7624, Raleigh, NC 27695-7624.

stained according to the applicable stain schedules. Stained sections were then photographed with a Wild photomicroscope fitted with a 35 mm camera.

Stain Schedules for Protein-bound and Free Amino Acids.

Coomassie Brilliant Blue G-250 (after Saio et al., 1983):

(a) Stain section for 2 min with dye solution by placing a drop of solution on the section. Use a 0.5% solution of Coomassie Brilliant Blue G-250 in 7% acetic acid and 50% methanol by dissolving 1 g of Coomassie Brilliant Blue G-250 in 100 mL of 100% methanol to which 100 mL of 14% acetic acid is added.
(b) Rinse the slide with 7% acetic acid and 50% methanol for a few seconds.
(c) Apply a few drops of 7% acetic acid and 50% methanol to prevent drying and add a cover slip.
(d) Protein-bound and free amino acids in the cytoplasmic network and protein bodies stain blue.

Orange G (after James and Tas, 1984):

(a) Stain section for 30 min with dye solution by placing a drop of solution on the section. Use a dye solution prepared by dissolving 0.1 g of Orange G in 100 mL of 1% acetic acid (pH 2.8).
(b) Rinse the slide for several seconds with 1% acetic acid (pH 2.8).
(c) Apply a few drops of 1% acetic acid to prevent drying and add a cover slip.
(d) Free terminal amino groups and free basic sidechains of lysine, arginine and histidine in the cytoplasmic network and protein bodies stain bright orange.

Diacetylbezene (after Pearse, 1985):

(a) Immerse sections for 5-10 min in 0.1 M veronal acetate buffer (pH 8.2).
(b) Immerse in 2% diacetylbenzene in 70% ethanol with an equal amount of 0.1 veronal acetate buffer (pH 8.2). The buffer should be added shortly before the beginning of the incubation. Leave for 30-60 min at room temperature.
(c) Wash briefly in buffer at pH 7.0 and then in three changes of distilled water.
(d) Apply a few drops of distilled water to prevent drying and add a cover slip.
(e) Amino groups in the cytoplasmic network and protein bodies stain red.

Stain Schedules for Sugars.

Alcian Blue - PAS Procedure (after Pearse, 1985):

(a) Immerse section for 2 hr in 1% Alcian Blue in 3% acetic acid.
(b) Rinse briefly in distilled water and then in 3% acetic acid. Rinse again with distilled water.
(c) Immerse section for 10 min in aqueous 1% periodic acid to oxidize.
(d) Wash in running water for 5 min.
(e) Immerse section in Schiff's reagent for 10 min.
(f) Wash in running water for 2 min.
(g) Rinse in three changes of 0.5% sodium bisulfite, 1 min in each.
(h) Wash in running water for 5 min.
(i) Apply a few drops of distilled water to prevent drying and add a cover slip.
(j) Sugars which are periodate-reactive and alcianophilic are localized in the cytoplasmic network and protein bodies by a bluish-purple end product.

Adaptation of Okamoto Method for Sugars (after Pearse, 1985): NOTE: Further treatment of glutaraldehyde vapor-fixed, freeze dried tissue blocks is required for this method before dehydration with absolute ethanol and infiltration with Spurr's resin. This treatment is described:

(a) Place glutaraldehyde vapor-fixed, freeze dried tissue blocks in methanol saturated with barium hydroxide for 24 hr.
(b) Immerse blocks for 5 min intervals in two changes of absolute ethanol.
(c) Immerse blocks in alcoholic silver nitrate for about 30 min (Prepare alcoholic silver nitrate by adding 90 mL of absolute ethanol to 10 mL of 20% aqueous silver nitrate.)
(d) Rinse blocks for 30 sec intervals in three changes of 96% alcohol.
(e) Reduce in alcoholic formalin (Prepare alcoholic formalin by adding 90 mL of absolute ethanol to 10 mL of 40% formaldehyde).
(f) Bring blocks through descending strengths of aqueous alcohol (75, 50, and 25% at 15 min intervals) to water.
(g) Immerse blocks for 1 min in 5% sodium thiosulfate.
(h) Wash blocks in distilled water and dehydrate for 5 min intervals in an ascending series of aqueous ethanol (25, 50, 75, and 95%) and finally in two changes of absolute ethanol.
(i) Sugars stain as a blackish stable end product in the cytoplasmic network and protein bodies.

Results and Discussion

Amino acids and sugars in peanut cotyledons have been postulated to combine during roasting (Newell *et al.*, 1967; Vercellotti *et al.*, 1995) to form the volatile compounds, which have been implicated as the character impact compounds of typical roasted peanut flavor. All three histochemical tests for protein-bound and free amino acids reported herein - (1) Coomassie Brilliant Blue G-250 (Fig. 1); (2) Orange G (Fig. 2); and (3) diacetylbenzene (Fig. 3) - localized amino acids in the protein bodies and cytoplasmic network. Both histochemical tests for sugars reported herein - (1) Alcian Blue - PAS procedure (Fig. 4) and (2) an adaptation of the Okamoto method (Fig. 5) - localized sugars in the protein bodies and cytoplasmic network as well. This localization of amino acids and sugar concentration primarily to the protein bodies suggests that future studies regarding amino acid, sugar, and roasted peanut relationships should be focused on the constituents within the protein bodies. Future advances in instrumentation should make it possible to conduct such experiments.

The results of this study, which indicate that amino

acids and sugars are both localized in the protein bodies, support the findings of Mason and Waller (1964). Mason and Waller (1964) reported from their fractionation experiments on roasted peanuts that the fraction containing primarily protein bodies was by far the most potent in typical roasted peanut aroma and tasted very much like peanut butter. This finding of the typical roasted peanut aroma in the protein body fraction is consistent with the histochemical localization in the present study of both necessary flavor precursors, namely amino acids and sugars, in the protein bodies.

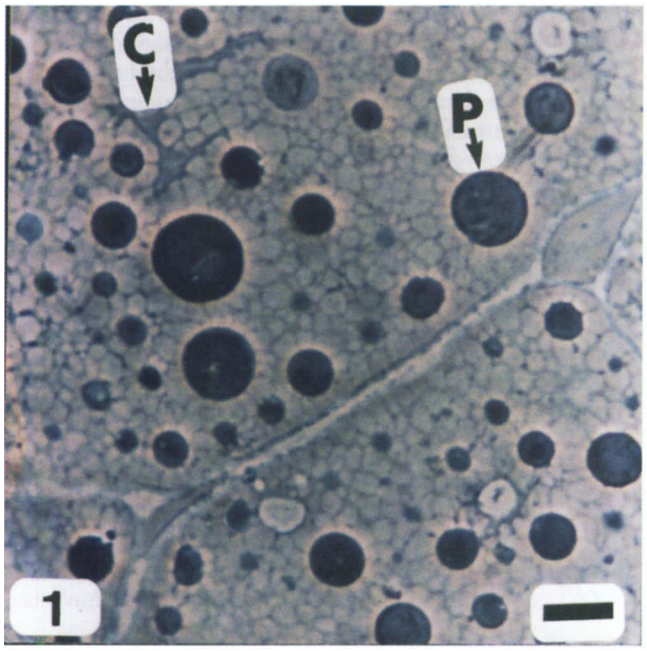

Fig. 1. Protein-bound and free amino acids stained blue with Coomassie Brilliant Blue G-250 in the cytoplasmic network (C) and protein bodies (P) in a peanut cotyledonary parenchymal cell. Bar = 8μm.

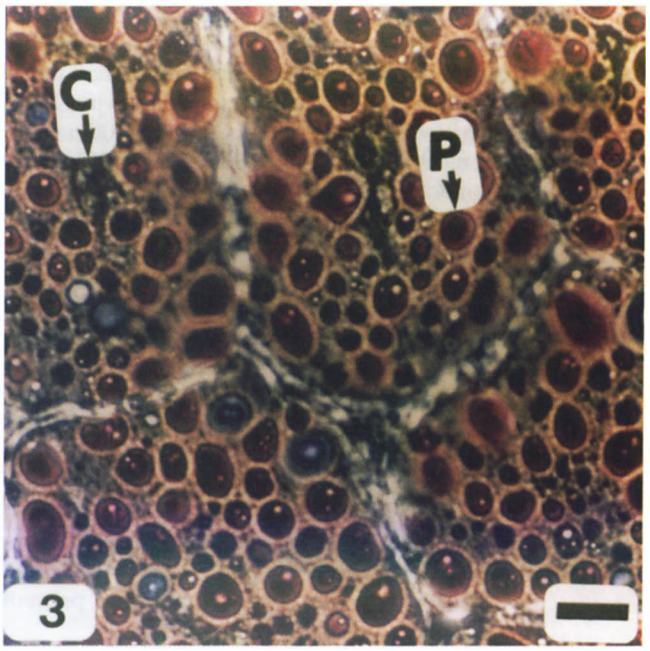

Fig. 3. Amino groups stained red with diacetylbenzene in the cytoplasmic network (C) and protein bodies (P) in a peanut cotyledonary parenchymal cell. Bar = 20μm.

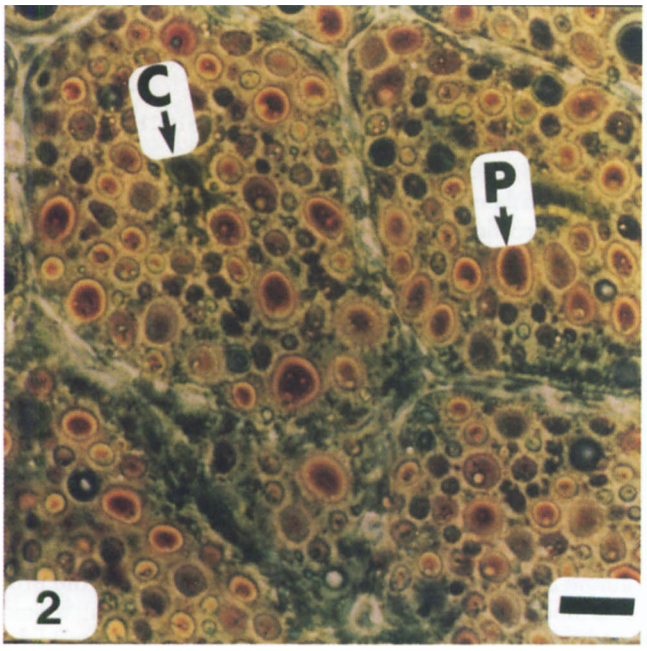

Fig. 2. Free terminal amino groups and free basic side-chains of lysine, arginine and histidine stained bright orange with Orange G in the cytoplasmic network (C) and protein bodies (P) in a peanut cotyledonary parenchymal cell. Bar = 20μm.

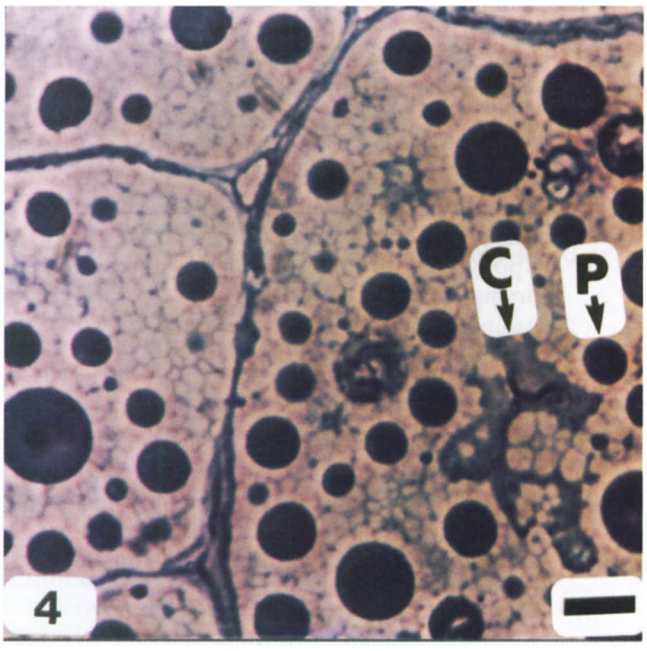

Fig. 4. Sugars stained bluish-purple after the Alcian Blue - PAS procedure in the cytoplasmic network (C) and protein bodies (P) in a peanut cotyledonary parenchymal cell. Bar = 8μm.

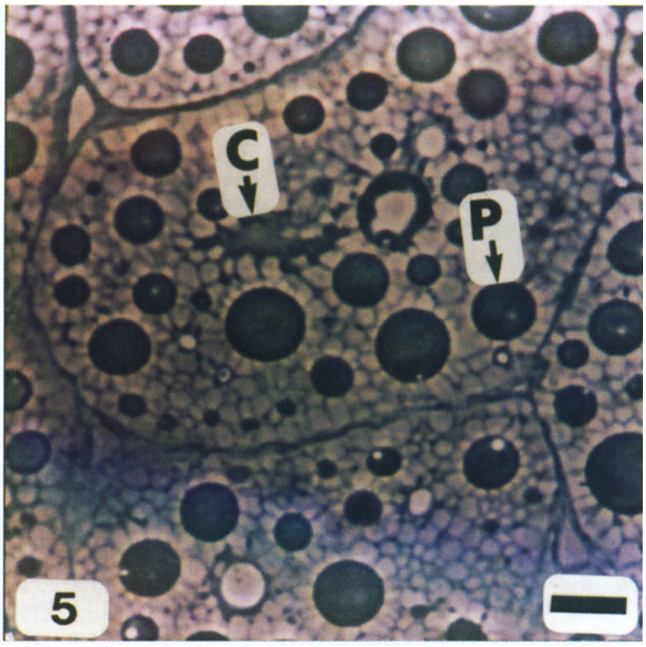

Fig. 5. Sugar stained black after treatment with the adaptation of the Okamoto method in the cytoplasmic network (C) and protein bodies (P) in a peanut cotyledonary parenchymal cell. Bar = 8μm.

Literature Cited

James, J., and J. Tas. 1984. Histochemical protein staining methods. Oxford University Press, Oxford, UK.

Mason, M.E., B. Johnson, and M.C. Hamming. 1996. Flavor components of roasted peanuts. Some low molecular pyrazines and a pyrrole. J. Agric. Food Chem. 14:454-460.

Mason, M.E., and G.R. Waller. 1964. Isolation and localization of the precursors of roasted peanut flavor. J. Agric. Food Chem. 12:274-278.

Newell, J.A., M.E. Mason, and R.S. Matlock. 1967. Precursors of typical and atypical roasted peanut flavor. J. Agric. Food Chem. 15:767-772.

Oupadissoon, C., and C. T. Young. 1984. Modelling of roasted peanut flavor for some virginia-type peanuts from amino and sugar contents. J. Food Sci. 49:52-58.

Pattee, H.E., and F.G. Giesbrecht. 1990. Roasted peanut flavor variation across germplasm sources. Peanut Sci. 17:109-112.

Pattee, H.E., F.G. Giesbrecht, and T.G Isleib. 1995. Roasted peanut flavor variation among U.S. genotypes. Peanut Sci. 22:158-162.

Pattee, H.E., F.G. Giesbrecht, and R.W. Mozingo. 1993. A note on broad-sense heritability of selected sensory descriptors in virginia-type *Arachis hypogaea* L. Peanut Sci. 20:24-26.

Pattee, H.E., T.G. Isleib, and F.G. Giesbrecht. 1998. Variation in intensity of sweet and bitter sensory attributes across peanut genotypes. Peanut Sci. 25:63-69.

Pattee, H.E., T.G. Isleib, F.G. Giesbrecht, and R.F. McFeeters. 2000. Investigations into sensory and chemical relationships in peanuts. J. Agric. Food Chem. 48: 757-763.

Pearse, A.G.E. 1965. Histochemistry - Theoretical and Applied. Volume 2. Churchill Livingstone, New York.

Pickett, T.A., and K.T. Holley. 1952. Peanut Roasting Studies. GA Agric. Exp. Sta. Tech. Bull. No.1.

Saio, K., Y. Nakano, and S. Uemoto. 1983. Microstructure of winged beans. Food Microstructure 2:175-181.

Spurr, A.R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastructure Res. 26:31-43.

Vercellotti, J.R., K.L. Bett, and K.S. Choi. 1993. Carbohydrates as a source of roasted peanut flavors and aromas, pp. 232-265. *In* G. Charalambous (ed.) 7th International Flavor Symposium, 1992, Samo, Greece. Elsevier Publishing Co., Amsterdam, The Netherlands.