of the harvested grain when the tannin content as measured by the vanillin assay is zero. Further studies are in progress to determine whether group II varieties are able to resist bird depredation at early stages of maturity.

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Chemical Nature of the Pigment of the Seed Coat of Guar (Cluster Bean, *Cyamopsis tetragonolobus* L. Taub)

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The pigment of the cluster bean (guar, *Cyamopsis tetragonolobus* L. Taub) seed coat was found to be a complex of ferric ions, galactose, gallic acid, and 2,3,4-trihydroxybenzoic acid.

Natural plant pigments are of diverse chemical structures (Bentley, 1960). Various phenolic compounds are constituents of plant pigments as reviewed by Singleton (1972). The presence of phenolic compounds in guar seed has previously been reported (Nagpal et al., 1971). The seed coat color of guar varies from black to dull white. Guar gum processing companies prefer seeds with a light colored seed coat. Hymowitz and Matlock (1967) studied the variations in seed coat color. They concluded that color variation is largely controlled by the environment. The chemical substances which produce the seed coat color are not known. This study was initiated to extract, purify, and identify the pigments.

MATERIALS AND METHODS

Procurement of Guar Seeds. Guar (*Cyamopsis tetragonolobus* L. Taub, var. FS 277) was grown on the farms of Panjab Agricultural University, Ludhiana, India. Seed samples were taken at 15-day intervals from flowering to maturity.

Extraction and Purification of Guar Seed Coat Pigment. The mature seeds ranging in color from light to dark brown were used for the extraction of pigment. The pigment was not extractable with organic solvents like methanol, ethanol, acetone, or ethyl acetate but was highly soluble in water. The whole guar seeds (2 kg) in small lots of 100 g each were immersed in glass-distilled water for 5 min at 15 °C. The supernatant was decanted off and filtered through Whatman No. 1 filter paper. The brown solution so obtained was lyophilized. Because it was not soluble in organic solvents it was possible to purify the water extract by several washings with methanol and acetone. The purified sample was then dried under vacuum. This purified compound was designated as G.

Extraction of Phenolic Acids. The samples (500 g) of guar seeds of various stages of maturity were extracted three–four times with 0.3 N HCl in methanol. The pH of the extract was adjusted to 8.3 with 5 N NaOH with constant stirring. A saturated solution of lead acetate was added according to the procedure of Walker (1962) and the light chocolate-grey precipitate obtained was filtered off and washed thoroughly with water until the filtrate became colorless. The precipitate was suspended in water and H_2S was passed through the suspension until the black precipitate of PbS ceased to form. The filtrate was freed from H_2S and concentrated by boiling it under reduced pressure on a water bath. The concentrate was extracted with ethyl acetate in a liquid–liquid extractor. The ethyl acetate fraction of each sample was stored in a refrigerator for chromatographic analysis.

Chromatographic and Spectral Analysis. Two-dimensional descending paper chromatography (PC) was carried out on 56 × 45 cm sheets of Whatman No. 1 filter paper (1 mm thick) employing the following solvents: (I)

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1-butanol–acetic acid–water (4:1:5, v/v), (II) 1-butanol–acetic acid–water (4:1:2.2, v/v), (III) acetic acid, 2% in water. Two-dimensional ascending thin-layer chromatography (TLC) was carried out on silica gel G coated plates employing the following solvents: (IV) benzene–methanol–acetic acid (45:8:4, v/v), (V) toluene–ethyl formate–formic acid (5:4:1, v/v), (VI) 1-butanol–ethyl acetate–2-propanol–acetic acid–water (35:100:60:35:30, v/v). The chromatograms were visualized in several ways: visible light, ultraviolet light (with and without ammonia vapors), diazotized *p*-nitroaniline/sodium carbonate (DPNA/Na_2CO_3), vanillin hydrochloride, bromocresol green, aniline phthalate, sodium metaperiodate–benzidine, neutral silver nitrate, and sodium nitroprusside–piprazine (Dawson et al., 1969); quinoline kojic acid reagent (Lederer and Lederer, 1957); and ferric chloride–potassium ferricyanide (Barton et al., 1952). The infrared (IR) spectra were taken in Nujol mull on a Perkin-Elmer 337 IR spectrophotometer and ultraviolet (UV) spectra in methanol on a Hilger-Watts UV spectrophotometer.

RESULTS AND DISCUSSION

Chromatographic Analysis of the Seed Coat Pigment. The pigment G was dissolved in water and paper chromatographed two dimensionally using solvents I and III, respectively. A number of chromatograms were obtained, and each one was visualized with the help of some of the specific spray reagents mentioned under the Materials and Methods section. It was observed that the spot G did not move at all in the first direction, and just streaked in the second direction. It was, therefore, thought that it might be better to hydrolyze it with acid and then chromatograph it. However, the chromatograms of unhydrolyzed G did indicate that the pigment was quite pure and it gave reactions with a number of these spray reagents which will be discussed along with the reactions of the hydrolyzed G.

Acid Hydrolysis of the Seed Coat Pigment and Its Analysis. The pigment G was hydrolyzed with 6 N HCl in a sealed test tube in vacuum for 16 h at 105 °C. The HCl was removed under vacuum by lyophilizing and the product obtained was labeled as GH. GH was not soluble in water. It was extracted with acetone and the extract was concentrated and labeled as GH–A. GH–A was paper chromatographed two dimensionally. A number of chromatograms were obtained and each one was visualized with the help of the specific spray reagents. Figure 1 shows the two spots revealed with ferric chloride–potassium ferricyanide reagent. Both the spots became straw-yellow with bromocresol green and light brown with DPNA/Na_2CO_3 and did not react with vanillin hydrochloride. The R_f values of spot 1 on descending PC were 0.57–0.62, 0.68–0.70, 0.45–0.48 in solvents I, II, and III, respectively, and on silica gel G TLC 0.22–0.25 and 0.42–0.45 in solvents IV and V, respectively. From these characteristics, spot 1 was tentatively identified as gallic acid. The cochromatography with the standard gallic acid was carried out on PC and TLC which confirmed it to be a gallic acid. The UV and IR spectra of this compound, which was eluted from the paper chromatograms by visualizing this spot under UV light as a violet spot, were recorded and found to be as expected for gallic acid. Spot 2 (Figure 1) gave R_f values as 0.67–0.70 and 0.55–0.56 on descending PC in solvents I and III, respectively, both values being higher than gallic acid. It appeared very faint tan in visible light and violet in UV light, which on exposure to ammonia changed to violet-blue. It turned yellow with bromocresol green which indicated it to be a phenolic acid. Its color reactions were similar to those of gallic acid with the spray

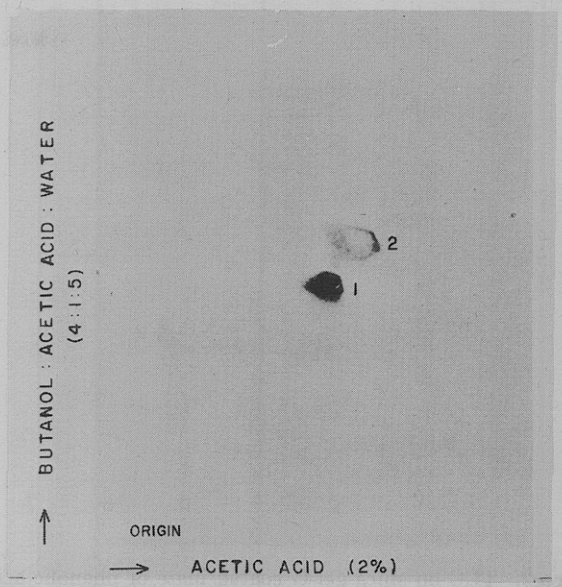

Figure 1. Two-dimensional PC of the acetone extract of HCl hydrolyzed guar seed coat pigment. It was visualized with ferric chloride–potassium ferricyanide reagent.

reagents used for spot 1 except with neutral silver nitrate solution in acetone. It turned grey, whereas gallic acid turned brown on spraying this reagent. The R_f values of spot 2 were almost similar to those of protocatechuic acid, another standard tried in solvents I, II, and III on PC, but it gave a yellowish tan color with 1% ethanolic ferric chloride, whereas gallic acid and protocatechuic acid gave a violet color. This spot was cut out from several paper chromatograms by visualizing it under UV light and eluted. A part of it was decarboxylated according to the procedure described by Karrer (1950) and identified on TLC in solvents IV and V as pyrogallol using standard pyrogallol as a marker. These evidences lead to the identification of spot 2 as 2,3,4-trihydroxybenzoic acid, an isomer of gallic acid. Gallic acid is a common constituent of plant phenolics (Haslam, 1966), whereas 2,3,4-trihydroxybenzoic acid seems to be of restricted distribution as its occurrence has been reported, so far, only in tea (*Camellia sinensis*) (Cartwright and Roberts, 1954).

Phenolic Acids of Guar Seed at Various Stages of Maturity. Figure 2 shows that the phenolic acids which are extractable with methanolic HCl and precipitable as lead phenolates were mainly the gallic acid and 2,3,4-trihydroxybenzoic acid in the mature guar seed. These were almost absent in the immature seed and their concentration increased with the age of the seeds.

Detection of Ferric Ions in the Pigment. A portion of the hydrolyzed guar pigment (GH) was taken in 1 N HCl in which it dissolved, giving yellowish green solution. For purification, it was reprecipitated with 1 N NaOH, washed with water, dried under vacuum, and designated as GH-B. GH-B was soluble in HCl but not in water or organic solvents which suggested that it was inorganic. A portion of GH-B was taken up in a crucible and heated on a flame. It was not affected up to 500 °C but melted at a slightly higher temperature to a yellow mass which was oxidized immediately to a grey solid mass. With 2% aqueous $K_4Fe(CN)_6$, GH-B in HCl gave a prussian blue color, which suggested that it was a ferric salt. GH-B in HCl was cochromatographed with a solution of ferric chloride as described by Lederer and Lederer (1957). Only one spot was observed on spraying with 8-hydroxyquinoline kojic acid reagent; this spot migrated coincidently with Fe^{3+} ions and therefore confirmed that it was a pure ferric salt. PC of

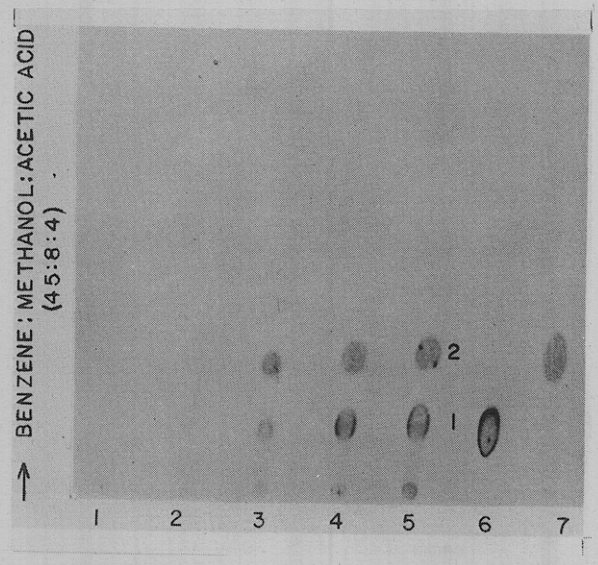

Figure 2. TLC on silica gel G coated plate of phenolic acid extracts of guar seeds at various stages of growth. (1–5) Stages of growth taken at 15-day intervals after flowering, (6) gallic acid, and (7) protocatechuic acid. Spots were visualized with the ferric chloride–potassium ferricyanide reagent.

the pigment (G) in solvent I on spraying with 8-hydroxyquinoline kojic acid reagent gave a black spot with a R_f value of zero, whereas PC of HCl hydrolyzed pigment (CH) gave a black spot with a R_f value of 0.71 (equivalent to ferric chloride marker), showing that the native pigment contained ferric ions in a bound form.

Detection of Bound Galactose in Pigment. G gave negative test with aniline phthalate, but positive test with sodium metaperiodate–benzidine, showing the presence of a sugar with a bound reducing group. In GH, galactose was identified by silica gel G (in 0.02 M boric acid) TLC in solvent VI with reference to a standard galactose spot. Quinic acid was found to be absent in the hydrolysate as tested by spraying with sodium nitroprusside piprazine.

Absence of Flavonoids, Anthocyanins, and Proteins. By visualisation of paper chromatograms of G and GH in various ways (visible light, UV light in the presence or absence of ammonia and spraying with specific reagents (Dawson et al., 1969), the flavonoids, anthocyanins, amino acids, and proteins were found to be absent. From the above evidences, it appears that the guar seed coat pigment consists of two phenolic acids, viz., gallic and 2,3,4-trihydroxybenzoic acids, ferric ions, and galactose. Though, in general, glucose is known to be the component of plant gallotannins (Haslam, 1966), in guar seed since galactomannin (a polysaccharide) constitutes 34–40% of the seed dry matter (Morimoto et al., 1962), it therefore appears that galactose may occur in guar gallotannins as well.

It was further observed that dull white or grey guar seeds on moistening and storage for 3–4 h at room temperature turn brown-black and resemble the other brown-black seeds present in the same lot. It appears that moistening facilitates the formation of brown pigment by allowing the phenolic acids and their derivatives to come in contact with the ferric ions complexed with the seed coat. Thus the variation in seed coat color arises from environmental conditions. This finding is of interest to industry since light seeds are preferred for manufacture of guar gum and guar meal. These results are in agreement with those of Hymowitz and Matlock (1967) who concluded from genetic trials that the variation in seed coat color was not genetic.

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