The pH-Sensitive Pigments in Pearl Millet[1]

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ABSTRACT

Millet flour-water pastes changed color reversibly from gray to yellow-green at alkaline pH and partially reversibly from gray to creamy-white, in the presence of acid. The methanol extract of the flour contained the pH-sensitive pigments, which were identified by paper chromatography as glucosylvitexin, glucosylorientin, and vitexin in the ratio of 29:11:4. These compounds are responsible for the intense yellow-green discoloration of the flour in the presence of alkali and may be responsible for the natural gray color of the peripheral endosperm of the grain. The methanol-extracted millet flour also contained a substantial quantity of alkali-labile ferulic acid (ALFA). The concentrations of total C-glycosylflavones and ALFA were 124 and 158 mg/100 g, respectively, in whole grains, but they decreased markedly on dehulling.

Pearl millets bleach from gray to creamy-white when the grains are soaked in low pH solutions (Reichert and Youngs 1979). In parts of Nigeria, sour milk or tamarind pods are traditionally used to provide the acidic environment. Dehulling of the grains expedites this bleaching process. This study was initiated to characterize and quantitate the pH-sensitive pigments in whole and dehulled millets. Reflectance spectroscopy was used to characterize light absorption properties of millet flour-water pastes, which aided in the determination of phenolics responsible for certain discolorations.

MATERIALS AND METHODS

Grain Samples and Processing

A commercial variety of millet grain (*Pennisetum typhoides*, early season variety) was obtained from Maiduguri, Nigeria, in June 1975. The hard red spring wheat used in this study was from the 1977 crop grown in Saskatchewan. Millet grain was dehulled in a Strong-Scott laboratory pearler (Strong-Scott Ltd., Winnipeg, Manitoba). Fines were separated on a 20-mesh screen to determine the percent of kernel removed by this process. Twenty-gram samples were ground 2 min in a CRC micro-mill (Chemical Rubber Company, Cleveland, OH) for reflectance measurements.

Reflectance Measurements

Dry flour measurements were taken on a Hitachi Perkin-Elmer Spectrophotometer equipped with a diffuse reflectance attachment (Reichert and Youngs 1976). Flour-water pastes (pH 1–12) for reflectance measurements were prepared by mixing flour with predetermined quantities of distilled water and HCl or NaOH as previously described (Reichert 1977). All pastes attained approximately the same smooth, creamy consistency.

Phenolic Identification

Phenolics in the methanol extract of millet were identified mainly by the methods of Mabry et al (1970). Millet flour (50 g), which had previously been extracted with petroleum ether (BP 58°C) and desolventized, was refluxed twice for 20 min with 200 ml of methanol. The combined extracts were reduced in volume under vacuum and taken to 200 ml with methanol. This extract was refrigerated at −10°C for two days to precipitate some sugars. A 15-ml aliquot was further reduced in volume to approximately 1 ml. Samples (20 µl) were chromatographed on Whatman 3MM paper by descending chromatography in 15% acetic acid in water (15% HOAc) and also in 3:1:1 t-butanol/acetic acid/H_2O (TBA). All spots, including the origin, were eluted from the 15% HOAc paper chromatogram with 2 ml of spectroscopic grade methanol for 10 min. As a reference, spots equal in size to the phenolic spots were cut from areas on the paper over which the solvent had passed and these were similarly eluted. A UV spectrum was obtained for each eluate immediately after addition of 2 drops of 1.09*M* sodium methoxide. The relative percentage contribution of each phenolic to the absorbance at 387 nm was calculated. Spectra were obtained on a Beckman model 25 spectrophotometer, using the diagnostic reagents and techniques described by Mabry et al (1970).

To prepare hydrolyzable quantities of the major flavonoids, the charcoal procedure of Mabry et al (1970) was used to free the extract from interfering sugars. From 800 g of defatted millet flour, 1.05 g of flavonoid-rich material was obtained. Compounds were separated and purified on Whatman 3MM paper by developing in 15% HOAc, removing the desired phenolics by methanol elution of the dried paper, and then rechromatographing with TBA (Reichert 1977).

Acid hydrolysis of the isolated compounds was accomplished by heating 1 mg of each compound in 5 ml of 2*N* HCl at 100° C for 45 min. Similarily, a mild hydrolysis was accomplished by heating each compound in 0.25*N* HCl for 20 min at 100°C. Each solution was evaporated (50°C) to dryness under vacuum, and the reaction products were dissolved in 0.2 ml of methanol. The resulting sugar was identified by co-chromatography on cellulose thin-layer chromatography (TLC) and visualized by spraying with p-anisidine HCl and heating at 120° C for 5–10 min (Pridham 1956). Flavonoid aglycones were separated with TBA and 15% HOAc by paper chromatography and identified by comparison to the R_f values and UV spectra (Mabry et al 1970).

An alkaline hydrolysis of methanol-extracted millet flour was performed by slowly adding 8 g of this flour to 200 ml of deaerated 2*N* NaOH and stirring at room temperature under N_2 for 4 hr (Steck 1967). The solution was neutralized to pH 3.0 with 12*N* HCl with ice cooling, and the volume was made up to 350 ml with H_2O. A 50-ml aliquot was taken, and 450 ml of methanol was added by drops to precipitate much of the solubilized starch and salt. After filtration, the volume was reduced under vacuum to approximately 1 ml with intermittent filtering to remove more salt. The major phenolic acid in this hydrolysate was identified by use of cellulose TLC and methods outlined by Steck (1967).

Phenolic Quantitation

The concentration of total C-glycosylflavones was determined on the basis of the absorbance (387 nm) of the crude methanol extract described above, adjusted to alkaline pH with 1.09*M* sodium methoxide. The concentration was calculated on the basis of the contribution of the C-glycosylflavones to the absorbance at 387 nm (from paper chromatography), a derived value for the extinction coefficient of 23,205, and the molecular weight of glucosylvitexin (Reichert 1977). Concentration is reported in terms of glucosylvitexin equivalents and is on a whole seed and dry weight basis, not a fat-extracted basis.

To determine the concentration of alkali-labile ferulic acid (ALFA), 2 g of the dried methanol-extracted flour was added to 100 ml of deaerated 2*N* NaOH, which was subsequently stirred at room temperature under N_2 for 4 hr. This suspension was neutralized to pH 2.0 with 12*N* HCl with ice cooling, and the volume was made up to 200 ml. Fifteen milliliters of this extract was added to

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20 ml of H_2O and then extracted twice with ether in a separatory funnel. The ether extracts were made up to 100 ml before measuring the absorbance at 317 nm. Acid-cleaned glassware must be used in the determination to avoid interferences. The concentration of ferulic acid was calculated on the basis of the contribution of ferulic acid to the absorbance at 317 nm (from cellulose TLC), the extinction coefficient of ferulic acid in ether (19,863), and the molecular weight of ferulic acid (Reichert 1977). The concentration of ALFA is reported on a whole seed and dry weight basis, not a fat-extracted and methanol-extracted basis.

RESULTS AND DISCUSSION

Effect of pH and Methanol Extraction on Millet Flour Color

Flour-water pastes prepared from whole millet flour at pH 1–4, pH 5–7, and pH 8–12 were creamy-white, gray, and yellow-green in color, respectively. A marked discoloration at alkaline pH is very common, but the bleaching effect observed at low pH is unusual.

Millet flour-water pastes absorbed more light at 400 nm than at 600 nm for the entire pH range (Fig. 1). Although the decrease in absorbance for both wavelengths was essentially equal for the pH change from 6.3 (natural pH) to 2.0, a pH change from 6.3 to 12 resulted in a much larger absorbance reading at 400 nm than at 600 nm. The flour color change that occurred on taking the pH to 10 was completely reversible, whereas the change occurring when the pH was adjusted to 2 was not reversible. A flour-water paste adjusted after 10 min from pH 2.0 to 6.3 had the absorbance readings at 400 nm and 600 nm illustrated by the horizontal lines in Fig. 1. These readings represent only 80 and 75% of the original absorbance difference at 400 and 600 nm, respectively. Acid treatment of millet appears to modify or dissociate some pigment in millet irreversibly.

The reflectance spectroscopic characteristics at high and low pH of flour-water pastes prepared from wheat flour, millet flour, and boiling methanol-extracted millet flour are compared in Fig. 2. The millet flour-water paste at pH 10.0 exhibited a sharp absorption maximum at approximately 390 nm (Fig. 2A). Methanol extraction of millet flour reduced the absorption maximum of the flour-water paste to a level comparable to that in wheat and shifted it to approximately 375 nm (Fig. 2B and C). This illustrates that methanol solubilized the compounds that are responsible for the marked yellow-green discoloration of the flour-water paste at alkaline pH (absorption maximum at 390 nm). A methanol extraction of cracked millet grain (8 hr, 25°C) demonstrated that methanol also solubilizes the natural gray pigmentation of the grain, which is concentrated in the peripheral region of the endosperm.

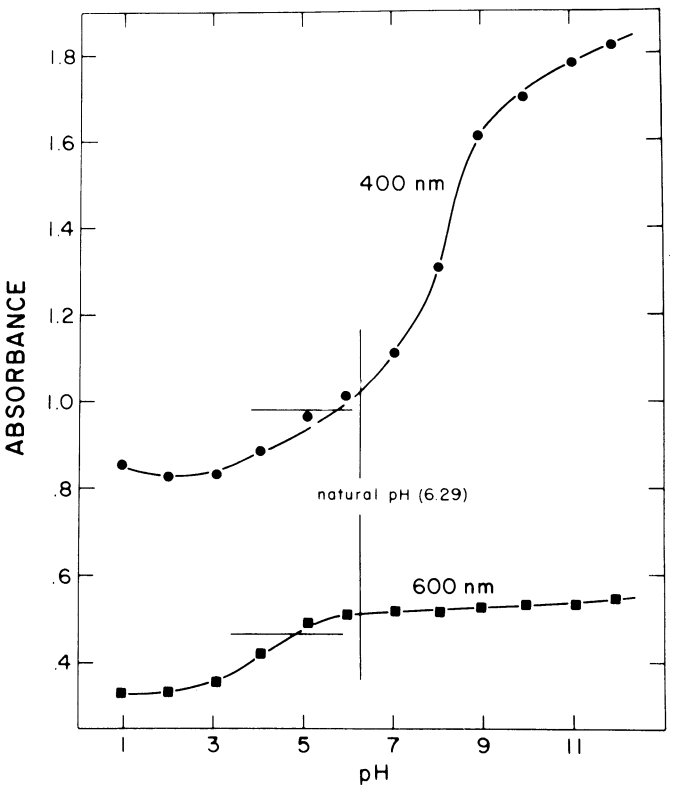

Fig. 1. Reflectance characteristics (400 and 600 nm) of millet flour-water pastes at pH 1–12. Horizontal lines represent reflectance measurements of a flour-water paste adjusted after 10 min from pH 2.0 to 6.3.

Characterization of Millet Pigments

The boiling methanol extract of millet flour was yellow-green at alkaline pH and exhibited a sharp absorption maximum at 387 nm. At low pH the extract was nearly colorless.

Direct paper chromatography of the boiling methanol extract with 15% HOAc separated the constituents, even though sugars present reduced the R_f values somewhat. Chromatography with TBA did not separate the constituents sufficiently. Six spots were observed on the 15% HOAc chromatograph at R_f 0–0.07, 0.13, 0.23, 0.37, 0.53, and 0.67. All spots were fluorescent blue under long wavelength UV light, except for R_f 0.23 and R_f 0.53, which were purple. All spots were eluted with methanol and a UV spectrum was taken immediately after addition of sodium methoxide. The relative percentage of each spot was 8.9, 2.7, 6.0, 15.0, 60.6, and 7.3%, respectively. On rechromatographing the spot at R_f 0.23 with 15% HOAc and TBA, this compound matched the R_f values and spectral characteristics given by Mabry et al (1970) for the C-glycosylflavone vitexin. The spot at R_f 0.53 was rechromatographed with 15% HOAc and TBA and separated into two constitutents with TBA at R_f 0.48 and R_f 0.32 in a ratio of 29:11, respectively. Acid hydrolysis (2*N* HCl, 45 min, 100°C) of the major constitutent (R_f 0.48, TBA), which was a pale yellow compound, yielded the known C-glycosylflavones vitexin and isovitexin (7:2) as the aglycones (Mabry et al 1970) and glucose as the only sugar. Similarly, acid hydrolysis of the minor constitutent (R_f 0.32, TBA), which was a yellow compound, yielded the known C-glycosylflavones orientin and isoorientin (7:2) as the aglycones (Mabry et al 1970) and glucose as the only sugar. A mild hydrolysis of R_f 0.48, TBA and R_f 0.32, TBA, in which most of the glycosides remained unhydrolyzed, yielded only vitexin and orientin, respectively, as the true aglycones of the compounds. Isovitexin and isoorientin produced by rigorous hydrolysis conditions were artifacts produced as a consequence of the Wessely-Moser rearrangement. The rearrangement is common with C-glycosylflavones (Chopin and Bouillant 1975) and has been shown to occur with vitexin (Mabry et al 1970, Seikel and Geissman 1957).

The glucose derivative of vitexin exhibited spectral characteristics in diagnostic reagents as follows: MeOH (nm) 270, 302sh, 333; + NaOMe 280, 330, 395; + $AlCl_3$ 276, 302, 346, 384; + $AlCl_3$/HCl 278, 303, 343, 384; + NaOAc 280, 305sh, 382; + NaOAc/H_3BO_3 271, 324, 344. Similarily, spectral characteristics of the glucose derivative of orientin were: MeOH (nm) 254, 268, 345; + NaOMe 270, 276sh, 336sh, 405; + $AlCl_3$ 273, 300sh, 330, 424; + $AlCl_3$/HCl 265sh, 273, 295sh, 354, 386; + NaOAc 270sh, 280, 330sh, 394; + NaOAc/H_3BO_3 263, 373, 430sh. The spectral characteristics of these glucose derivatives are nearly identical to those reported for the aglycones (Mabry et al 1970). This property is diagnostic for sugar derivatives of C-glycosylflavones in which the sugar is glucosidically attached to the sugar moiety of the C-glycosylflavone (Chopin and Bouillant 1975).

Two glucose units per molecule of vitexin and orientin derivative was assumed on the basis of relative R_f values. In 15% HOAc, apigenin (no sugar group), vitexin (1 sugar group) and rhamnosylvitexin (two sugar groups) have R_f values of 0.11, 0.29, and 0.72, respectively (Mabry et al 1970). Glucosylvitexin and glucosylorientin have R_f values of 0.72 and 0.66, respectively, in 15% HOAc, indicating that they each incorporate two sugar groups, the C-C bound glucose as well as a single glucosidically attached glucose molecule.

The structures of glucosylvitexin and glucosylorientin can therefore be assumed to be as shown in Fig. 3. These compounds and vitexin account for 66.6% of the absorbance at 387 nm of the boiling methanol extract adjusted to alkaline pH.

Glucosylvitexin, glucosylorientin, and vitexin have their maximum absorption at 395, 405, and 395 nm, respectively, in MeOH + NaOMe. These compounds are mainly responsible for the yellow-green color of millet flour at alkaline pH and for the absorption maximum of the flour-water paste (pH 10) observed at 390 nm (Fig 2A). These compounds also may be responsible for the natural gray pigmentation in the peripheral endosperm of the grain. In vitro, however, these compounds are not gray. Many phenolics in vivo do not appear to be the same color as the pure compounds (Singleton 1972). The gray color of millet grain may be caused by several factors: chelation of the phenolics in vivo with copper, iron, aluminum, or other metal ions; copigmentation effects, which enhance pigments; or pH effects, which change the degree of ionization of the phenolic (Singleton 1972). More work is required to definitively establish the nature of the gray pigment in millet.

To investigate which phenolics further contributed to the acid-base sensitivity of methanol-extracted millet flour (Fig. 2B) and particularily to the absorption maximum at 375 nm at alkaline pH, this flour was further base-hydrolyzed. Three phenolics were released. Ferulic acid constituted approximately 89.8% of the total, based on the UV spectral absorption at 317 nm of eluted spots. Carbohydrate or amino acid esters of ferulic acid at alkaline pH absorb light in the region near 375 nm. For example, the glucose and quinic ester derivatives of ferulic acid absorb at 383 and 375 nm, respectively, in ethanol containing NaOH (Steck 1967). It is probable that ferulic acid is bound in a like manner in millet flour

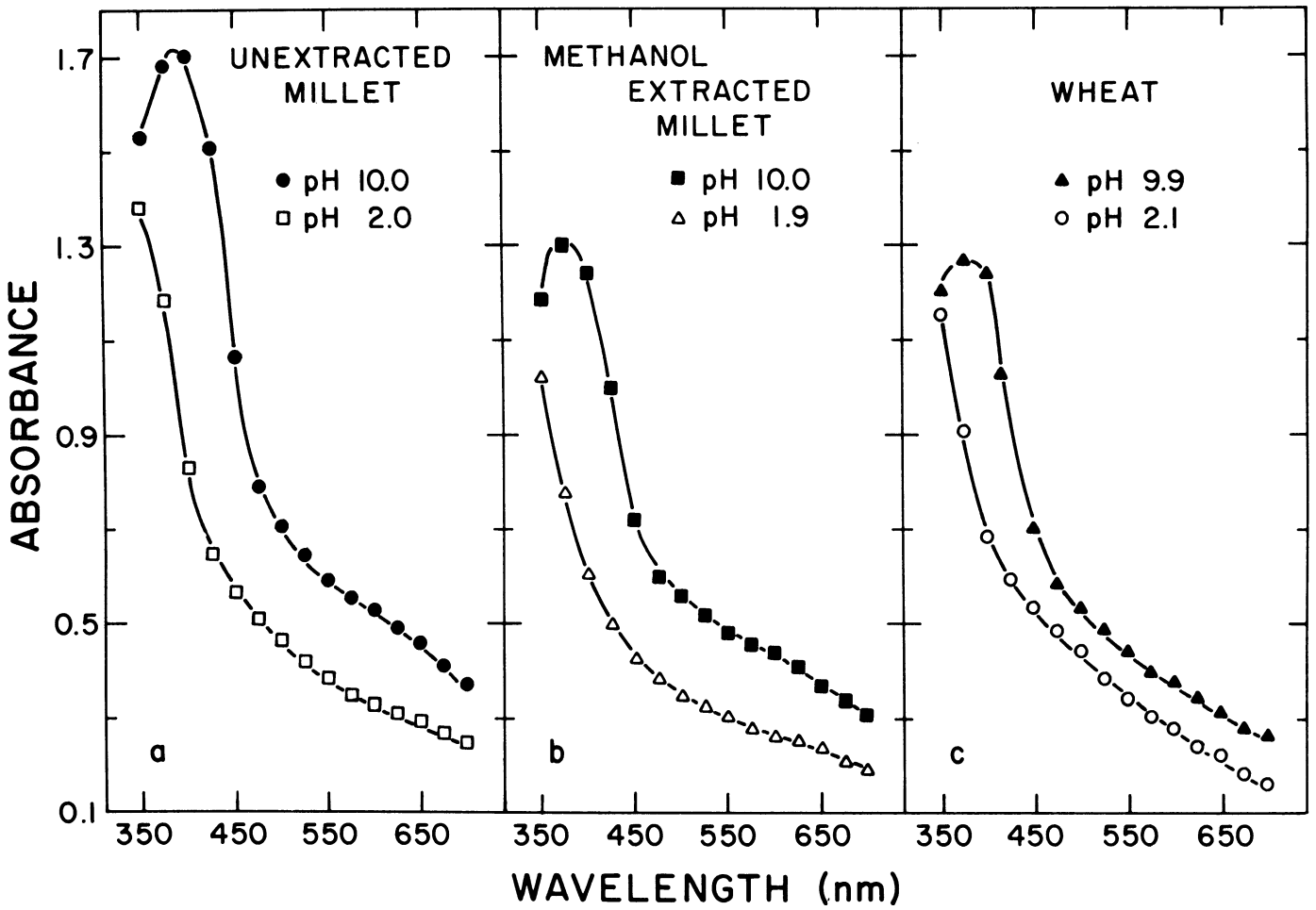

Fig. 2. Reflectance wavelength scans of unextracted millet, methanol-extracted millet, and wheat flour-water pastes at acid and alkaline pH.

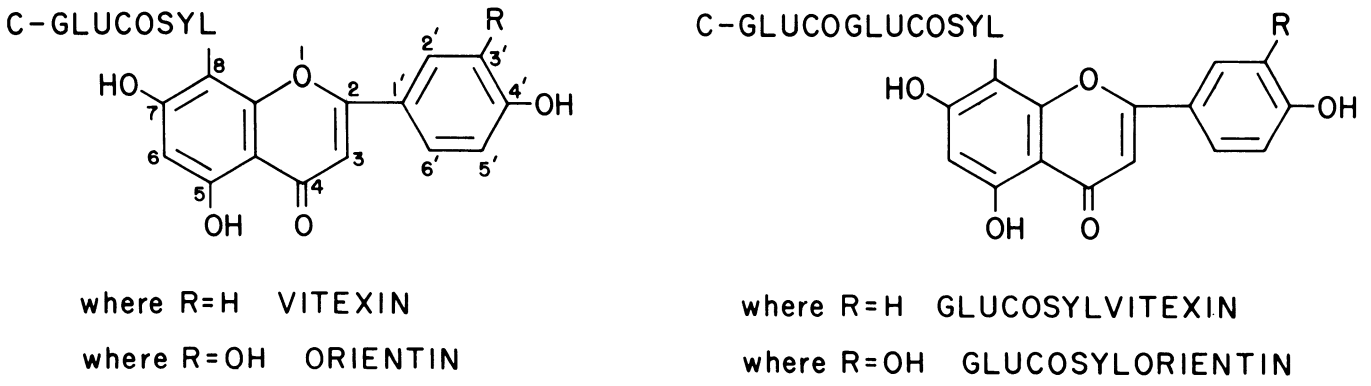

Fig. 3. Structures of glucosylvitexin, glucosylorientin, and vitexin present in the ratio of 29:11:4, respectively, in pearl millet. No orientin was detected.

and that this type of linkage contributes to the maximum at 375 nm observed in the flour-water reflectance spectrum of methanol-extracted millet flour at alkaline pH.

Concentration of Millet Pigments

Whole millet contained 124 mg/100 g of C-glycosylflavones (measured as glucosylvitexin equivalents) and 158 mg/100 g of ALFA. Concentrations of these phenolics were markedly decreased in dehulled grains (Fig. 4). After nearly 50% of the kernel was removed with the laboratory pearler, the concentrations of both C-glycosylflavones and ALFA in the dehulled grains were about 20% of the concentrations in the whole grains. The correlation coefficient between total C-glycosylflavone or ALFA concentration and dry flour reflectance of pearled samples at 450 nm was 0.994 and 0.991, respectively. This indicates that the color of millet flour from pearled samples is highly correlated with the phenolics identified in the grain.

Concentrations of the C-glycosylflavones and ALFA are much higher in the hull fractions than in the dehulled grains. If 9.1% dehulling were achieved in a mill, the concentrations of C-glycosylflavones and ALFA in the hull fraction would be 392 and 593 mg/100 g, respectively, by calculation from Fig. 4. This concentration of total phenolics approaches 1% and as such may have some nutritional significance when hull fractions are used as animal feed.

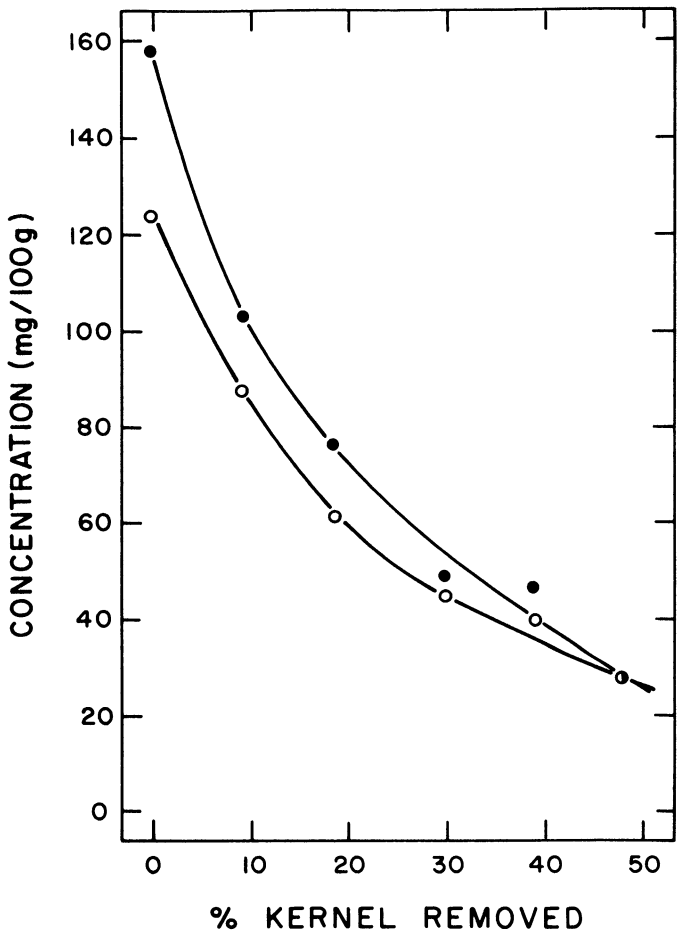

Fig. 4. Concentrations of phenolics in dehulled millet grain. (o) Total C-glycosylflavones. (•) Alkali-labile ferulic acid (ALFA) of the methanol-extracted flour.

The concentration of C-glycosylflavonoid apparently has been reported only once for seeds of the family Gramineae (King 1962). However, Chopin and Bouillant (1975) reported that there are many instances in which C-glycosylflavonoids have been simply identified in this family, particularly in the leaves. King (1962) reported that commercial wheat germ contained 0.2–0.3% of two C-glycosylflavones, although no flavonoid components could be detected in either the bran or endosperm fractions. If this wheat germ material comprised 10% of the whole seed, the concentration of the C-glycosylflavones in whole wheat would be about 0.02–0.03%, or five times less than in whole millet. The concentration of ALFA in millet is about 1.5 times that in barley (0.105% ALFA) as reported by Van Sumere et al (1972).

In summary, the contribution of C-glycosylflavones to the color of millet flour-water pastes at high pH has been illustrated; these compounds may be responsible for the natural gray color of the grain. Further investigation will be required to determine whether there is any biochemical, functional, or nutritional significance of the high concentration of C-glycosylflavones and ALFA in the peripheral regions of pearl millet grain.

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