Relative Degree of Polymerization of Sorghum Tannin during Seed Development and Maturation

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Changes in the degree of polymerization of condensed tannins in extracts of developing sorghum seed can be estimated from the ratio of total flavan-3-ol residues (estimated by formation of anthocyanidins in HCl/butanol) to terminal flavan-3-ol residues (estimated by color production in the vanillin reaction carried out in glacial acetic acid). The degree of polymerization of the tannin extractable from several high-tannin, bird-resistant, sorghum cultivars remains constant and relatively low throughout seed development in the field. After the seeds are dried, even in grain samples that are quite immature, the relative degree of polymerization increases severalfold to values characteristic of mature dry grain. Other parameters related to the degree of polymerization change similarly on drying. These results suggest that bird repellency, which is maximal in immature undried grain, may be due to relatively short oligomers as well as nontannin phenolics. If this is the case, a significant improvement in the nutritional quality of high-tannin sorghum might be observed if it were possible to inhibit the polymerization during drying of the seed.

"Bird-resistant" grain sorghums contain condensed tannins (proanthocyanidins, oligomers of flavan-3-ols) that are thought to account for their bird-repellent properties (McMillian et al., 1972; Bullard et al., 1980; Hoshino and Duncan, 1981a). The amount of tannin that can be extracted from the seed and chemically assayed, as well as the bird repellency of the extracts (Bullard et al., 1981), changes considerably during the process of seed maturation. The tannin content, either on a per seed or on a dry weight basis, reaches a maximum early in the maturation process and in mature seed is usually reported to decline to levels that are different for different cultivars (Davis and Hoseney, 1979; Chavan et al., 1979; Price et al., 1979; Glennie et al., 1981; Bullard et al., 1981).

Goldstein and Swain (1963) suggested that the decline in tannin and in the associated astringency of fruits on ripening is due to an increase in the degree of polymerization of tannins. Likewise, decreases in assayable proanthocyanidins of sorghum on maturation have been ascribed to increased polymerization (Gupta and Haslam, 1978; Bullard et al., 1980).

Accurate measurement of the degree of polymerization of oligomeric proanthocyanidins is a formidable task. Czochanska et al. (1980) have determined molecular weights and other structural information for proanthocyanidin oligomers using ^{13}C NMR, but this technique involves isolation and purification of the tannin to be analyzed and is therefore not suitable for routine analysis of large number of samples. Bullard et al. (1981) interpreted changes, during seed development, in the ratio of two independent chemical assays for tannin in terms of increased degree of polymerization.

We have recently described assay conditions in which the vanillin reaction, widely used as an assay for condensed tannins, apparently measures only a single terminal flavan-3-ol unit in oligomers containing from 1 to 6 or more of these units (Butler et al., 1982). The average degree of polymerization of the tannin oligomers can be estimated in unpurified extracts by combining this adaptation of the vanillin reaction with an assay that detects all of the flavan-3-ol residues present, whether terminal or not. We have chosen to use the formation of anthocyanidin pigments from proanthocyanidins (Gupta and Haslam, 1980) to estimate total flavan-3-ol residues. The problems associated with carrying out this reaction on complex mixtures (Lewak, 1968) should not prevent its applicability in determining relative values in samples differing only in degree of maturity. Unlike the vanillin reaction, this assay does not detect flavan-3-ol monomers such as catechin. Thus, the degree of polymerization, as determined by the anthocyanidin formation/vanillin product ratio, is underestimated, especially in extracts rich in catechin and/or epicatechin. We make no attempt, therefore, to determine the absolute degree of polyermization of flavan-3-ols in the seed extracts. Instead, we have estimated the *relative* degree of polymerization throughout the process of development and drying for several high-tannin sorghums.

EXPERIMENTAL PROCEDURES

Materials. Sorghum samples were grown during the 1981 crop year at the Purdue University Agronomy Farm, under the direction of Dr. John Axtell. Immature seed samples were collected early in the morning by cutting the rachis with attached seeds from 8 to 10 representative panicles. Fresh samples were stored in a refrigerator for up to 3 h until extracts were prepared. Portions of each sample (rachis with seeds still attached) were dried at room temperature (23–26 °C) for 2 weeks before extraction and assay.

Extraction. Representative 25-seed samples, before and after drying, were ground in 10 mL of diethyl ether on a Polytron PT 10-35 homogenizer with a PT 20 probe generator (Brinkmann Instruments, Westbury, NY) at a setting of 4–6, in repetitive short bursts to minimize heating. Ether does not extract significant amounts of polyphenols but does remove lipids that interfere in the protein precipitation assay. After low-speed centrifugation the ether was discarded and the residue was reground in 10 mL of methanol and mixed for 15 min on a shaker. After centrifugation, the residue was reextracted with methanol and the two methanol extracts were combined for analysis. In order to obtain any tannin (type II) that does not extract into methanol but that does extract into acidic methanol (Price et al., 1978), the residue was further extracted twice with methanol containing 1% (v/v) concentrated HCl, and these two acidic methanol extracts were combined for analysis.

Assays. Total phenolic content of the extracts was measured by a modification of the Prussian blue assay of Price and Butler (1977). Samples (0.5 mL) were diluted with 30 mL of H_2O, and 3.0 mL of 0.05 M $FeCl_3$ in 0.1 N HCl was added. At t_0, 3.0 mL of 0.008 M $K_3Fe(CN)_6$ was

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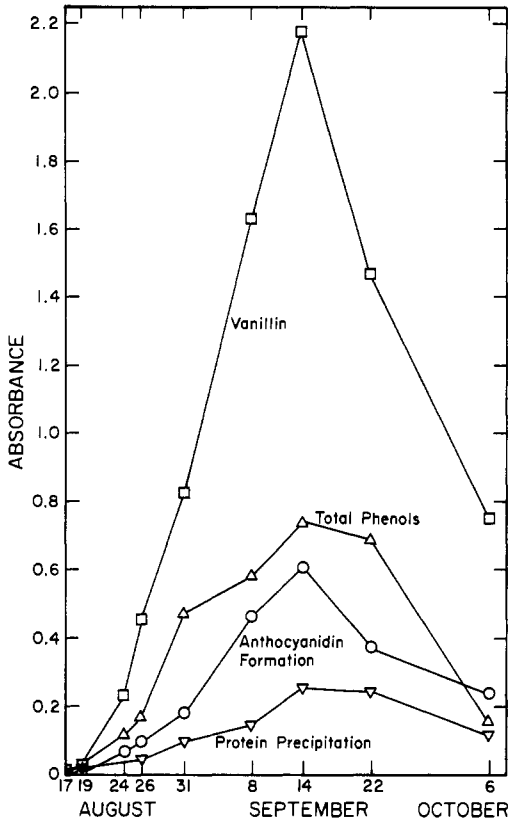

Figure 1. Analyses of fresh (undried) seed of sorghum BR 64 throughout seed development and maturation. Analyses of methanol extracts were carried out as described under Experimental Procedures.

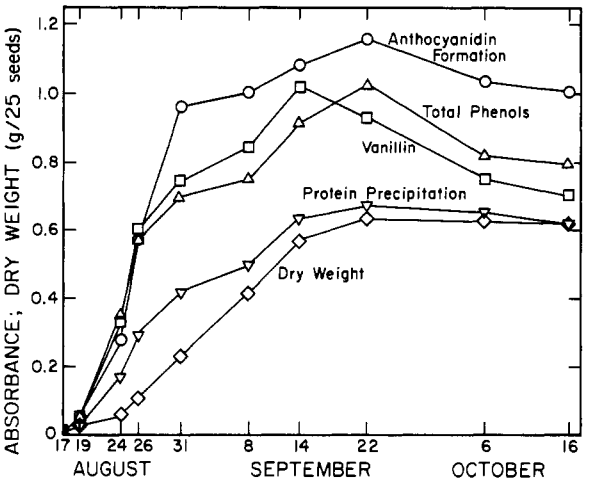

Figure 2. Analyses of seed of sorghum BR 64 (same samples as in Figure 1) that were dried at room temperature 2 weeks before extraction with methanol and assay.

added, and 20 min later the absorbance at 720 nm was determined spectrophotometrically against water. A methanol blank was run with each series.

Protein precipitating capacity was determined as described by Hagerman and Butler (1978), with the addition of the methanol wash of the precipitate as suggested by Bullard et al. (1981).

The vanillin assay was carried out with glacial acetic acid, rather than methanol, as the solvent (Butler et al., 1982). Methanol extracts of sorghum seed were diluted with 4 volumes of glacial acetic acid, and 2.0 mL of the diluted extract was assayed.

For measurement of anthocyanidin formation, 0.5-mL samples of extract were added to 7.0 mL of 30% (v/v) concentrated HCl in 1-butanol or to 15% (v/v) 0.1 N acetic acid, 15% (v/v) methanol, and 70% 1-butanol. No anthocyanidin formation occurs in the latter solvent, which serves as a blank to correct for pigments present in the extract. After being mixed, the samples are left at room temperature for 1 h in order to form the unstable anthocyanidin pigment characteristic of flavan-4-ols (Watterson and Butler, 1983). After the absorbance at 550 nm is read, the sample (but not the blank) is then heated on a boiling water bath for 1.5 h. Under these conditions flavan-3-ol oligmers are converted to anthocyanidins (and other products), and the unstable anthocyanidin pigment formed from flavan-4-ols is completely destroyed (Watterson and Butler, 1983). After being cooled, anthocyanidins are measured by their absorbance at 550 nm.

RESULTS

Amounts of Polyphenols during Maturation: Effect of Drying. A standard high-tannin bird-resistant hybrid, BR 64, was sampled throughout seed development and maturation and assayed for polyphenols by the four independent techniques. The results obtained on samples extracted immediately after collection, without drying, are shown in Figure 1 on a per seed basis. Half-anthesis occurred about Aug 9, and frost terminated growth on Oct 6. The patterns of changes in tannin content during seed development were similar in the vanillin and anthocyanidin formation assays, which are used to estimate the degree of polymerization of the tannin, as well as in assays for total content of phenolic groups and protein precipitating capacity. All four assays showed a maximum on Sept 15, with a subsequent decline as the seed matured. These results are in agreement with our earlier observations obtained under somewhat different conditions (Price et al., 1979).

However, the results of these assays are strongly dependent upon the extent and manner of drying the samples before grinding and extraction (Price et al., 1978; Hoshino and Duncan, 1981b; Butler, 1982). This effect is illustrated in Figure 2. Samples and assay techniques are the same in Figure 1 and 2; the only difference is that the samples in Figure 2 were dried at room temperature before assaying.

Comparison of the protein precipitation, total phenols, and anthocyanidin formation assays in Figures 1 and 2 reveals that the amount of extractable tannin per seed *increases* substantially on drying. We have observed that even in mature grain, tannin in moist seeds is difficult to extract (Butler, 1982), so this increase in tannin on drying of fresh immature seeds was not unexpected.

In contrast to the other assays, values for the vanillin assay *decrease* drastically on drying samples obtained midway through the development maturation process. Both early and later samples gave comparable values in fresh and dried seeds.

Degree of Polymerization. The results with the vanillin assay, which show a large *decrease* on drying, are clearly different from the *increase* on drying observed with the other three assays. In order to interpret this effect of drying, it is necessary to recall that flavan-3-ol monomers such as catechin and epicatechin give a positive test in the vanillin assay but are negative in the protein precipitation and anthocyanidin formation assays. Polymerization of monomers to tannin does not involve phenolic hydroxyls (Gupta and Haslam, 1978) so that assay for total phenols should be unaffected by polymerization. In contrast, the polymerization product, tannin, is positive in the protein precipitation and anthocyanidin formation assays. Furthermore, polymerization results in a diminished reaction

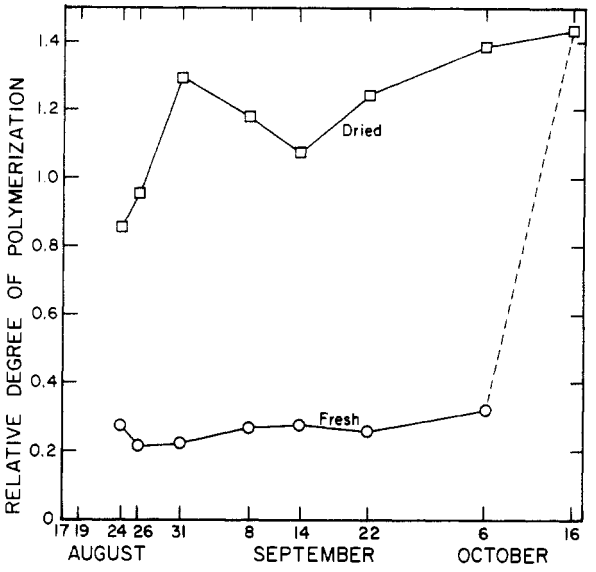

Figure 3. Relative degree of polymerization during seed development and maturation. Relative degree of polymerization is defined as the ratio of absorbance in the vanillin assay (using glacial acetic acid as the solvent) to the absorbance in the anthocyanidin formation assay. Data were taken from Figures 1 and 2.

with vanillin, because under the conditions employed here, only a single terminal flavan-3-ol residue of each polymeric molecule will react (Butler et al., 1982). Thus, comparison of the data in Figures 1 and 2 suggests that the fresh immature seeds contain large amounts of flavan-3-ol monomers and/or oligomers that are too short to precipitate protein and that during drying these materials further polymerize to form tannins. Similar results suggesting a large increase in the degree of polymerization during drying of immature grain were obtained with several other high-tannin sorghum cultivars: Savanna III, IS 6881, IS 15067, and IS 4225.

The relative degree of polymerization throughout maturation, calculated as the ratio of the vanillin absorbance (due to *terminal* flavan-3-ol residues) to the absorbance of the anthocyanidins formed (due to *total* oligomeric flavan-3-ol residues) is presented in Figure 3 for seeds extracted both while fresh and after drying. The relative degree of polymerization increased 3–4-fold on drying at all stages of seed development. The increase in tannin content as measured by protein precipitation and anthocyanidin formation (Figures 1 and 2) is due to lengthening of tannin molecules during the drying process and/or to increased extractability on drying, as noted above. Freezing, boiling, and drying at high temperatures have drastic effects on the assayable tannin of immature grain (Price et al., 1979; Hoshino and Duncan, 1981b) and may reflect enzyme involvement in tannin polymerization and/or membrane partitioning of flavan-3-ols from cellular components to which they otherwise bind and become unextractable (Morrall et al., 1981).

Interestingly, the relative degree of polymerization changed much less throughout maturation, for both fresh and dried seeds, than it changed on drying of fresh seeds at any particular time (see Figure 3). The relative degree of polymerization was not calculated for the earliest samples (Figures 1 and 2), because of the uncertainty in the small absorbance values, and for the last samples, because no fresh tissue was present at maturity. There is no significant increase in the relative degree of polymerization in fresh tissue, the physiologically significant form, throughout the development process. Results similar to those reported here for BR 64 were obtained with the other high-tannin sorghums assayed.

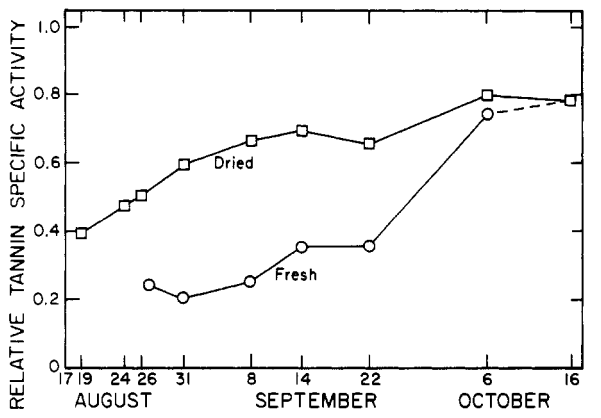

Figure 4. Relative tannin specific activity during seed development and maturation. Relative tannin specific activity is defined as the ratio of absorbance in the protein precipitation assay to the absorbance in the assay for total phenols. Data were taken from Figures 1 and 2.

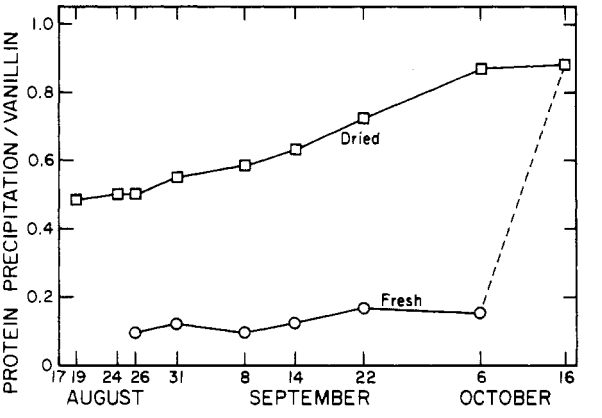

Figure 5. Relative protein precipitating capacity per terminal flavan-3-ol unit during seed development and maturation. The ratio of absorbance in the protein precipitation assay to the absorbance in the vanillin assay is plotted vs. collection date. Data were taken from Figures 1 and 2.

Other Parameters Related to the Degree of Polymerization. We have previously defined the ratio of the protein precipitating capacity to the total phenol content as the tannin specific activity (Hagerman and Butler, 1980). If flavan-3-ol monomers and oligomers too short to precipitate proteins polymerize to tannins on drying of immature seeds, the resulting increase in protein precipitating capacity should be reflected in a corresponding increase in tannin specific activity, because the total phenol content should be the same in both cases. The data in Figure 4 show that the relative tannin specific activity of dried seeds is greater than that of fresh seeds, as expected. Moreover, a significant increase in relative tannin specific activity is observed throughout maturation, especially in fresh seeds.

Similarly, polymerization of flavan-3-ol monomers should lead to an increase in the protein precipitating capacity and a decrease in the concentration of terminal flavan-3-ol residues as measured by the vanillin assay. Thus, the ratio of absorbance in the protein precipitation assay to absorbance in the vanillin assay would show a large increase on drying the seed, if polymerization occurs. As seen in Figure 5, the expected increase on drying is observed. In fresh tissue the protein precipitating capacity per terminal flavan-3-ol residue changes relatively little, but in dried seeds it almost doubles during maturation.

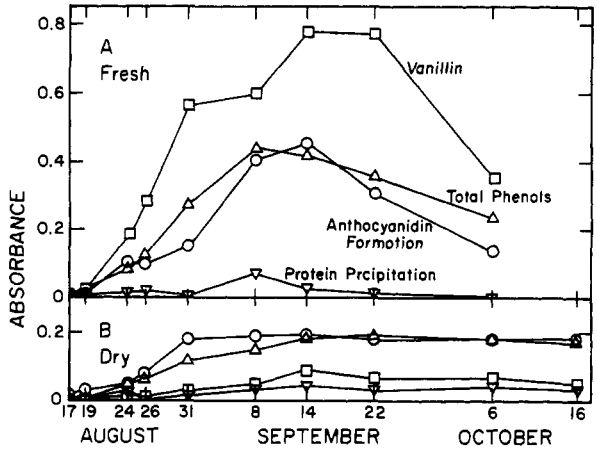

Figure 6. Polyphenols not extractable in methanol but extractable in acidic methanol. The residue of sample extracted in methanol (results in Figures 1 and 2) were extracted with 1% (v/v) HCl in methanol and analyzed as in Figures 1 and 2. Symbols have the same meaning in parts A (fresh tissue) and B (dried tissue).

This change, like the relative degree of polymerization (Figure 3), is small compared to the change on drying immature seeds.

Tannin Extractable Only in Acidic Methanol. After extraction with ether and twice with methanol, additional polyphenols were obtained by two further extractions of the residue with acidic methanol. The amount of this material was much greater in fresh than in dried seeds (Figure 6), in contrast to the methanol-extractable material (Figures 1 and 2), suggesting that polyphenols difficult to extract from fresh tissue with methanol constituted much of the tannin extracted by the acidic methanol. The relative degree of polymerization of the material extracted in acidic methanol was 2–3-fold higher than that of the corresponding methanol extracts (Butler, 1982). This is consistent with the suggestion by Bullard et al. (1981) that tannins extractable only in acidic methanol are more highly polymerized than those extractable in methanol. The lower relative degree of polymerization of the methanol extracts may also be due to the presence of most of the flavan-3-ol monomers in the methanol extracts, which preceded the extraction with acidic methanol. As in the methanol extracts, the relative degree of polymerization of the acidic methanol extracts increases severalfold on drying the immature seeds (Butler, 1982).

DISCUSSION

The data presented here clearly show that immature grains of high-tannin sorghum contain flavan-3-ols capable of polymerization to tannins, in agreement with the observations of Gupta and Haslam (1978). When flavan-3-ol polymerization is complete and seeds have matured and become dry, even bird-resistant high-tannin sorghums suffer bird damage (Tipton et al., 1970; Price and Butler, 1982). Thus, relatively short flavan-3-ol oligomers may contribute to the bird repellency of high-tannin sorghums. Bullard et al. (1980) reported that a nontannin fraction of sorghum polyphenols, which might include short oligomers, was more repellent to *Quelea*, the destructive African weaver finch, than was the tannin fraction that would include the more highly polymerized flavan-3-ols. It may even be possible to diminish antinutritional effects and prolong bird resistance by somehow interfering with the polymerization process.

The relative degree of flavan-3-ol polymerization, as I have defined and measured it, remains relatively constant in sorghum grain throughout seed development. When seed development is terminated by removal from the plant, by normal maturation, and probably also by frost, the relative degree of polymerization increases dramatically. In the sorghum variety investigated by Haslam (1977), this process was accompanied by development of luteolinidin pigmentation. The large change on drying immature seeds underscores the importance of rapid extraction of this tissue after collection, in order to draw conclusions about the degree of polymerization in the growing seed. These results are in agreement with previous conclusions that the degree of polymerization of the tannin of mature seed is greater than that of immature seed (Gupta and Haslam, 1978; Bullard et al., 1981) and differ mainly in that I suggest that much of the polymerization occurs late and rapidly, rather than gradually throughout the season. This pattern minimzes tannin interference in metabolic activity associated with seed development.

The relative protein precipitating power per flavan-3-ol-containing molecule (Figure 5) shows a pattern similar to that seen in the relative degree of polymerization (Figure 3), in agreement with the interpretation suggested above. The pattern for the relative tannin specific activity (Figure 4) is less similar but nevertheless is consistent with an increase in degree of polymerization on drying.

The relatively small proportion, in mature, dry grain, of tannin that extracts into acidic methanol but not into methanol (Figures 2 and 6B) identifies BR 64 as a group III sorghum (Price et al., 1978). Sorghum lines in which a high proportion of the tannin is extractable only in acidic methanol are classified as group II types (Price et al., 1978). Such sorghums are of considerable interest because the nutritional problems associated with conventional (group III) high-tannin sorghums have been reported to be absent in group II types (Oswalt, 1975). Tannins from group II sorghums have somewhat different characteristics than those from group III sorghums (Bullard et al., 1981). The properties of group II sorghum tannins will be described in a subsequent report.

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Determination of Phytic Acid in Foods by High-Performance Liquid Chromatography

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Our recently reported high-performance liquid chromatographic method for the determination of phytic acid [*myo*-inositol 1,2,3,5/4,6-hexakis(dihydrogen phosphate)] has been examined in greater detail and adapted to the analysis of foods. This slightly modified procedure consists of extraction of sample with 0.5 N HCl, filtration, dilution with water, passage of extract over anion-exchange resin AG 1-X8, and elution of phytic acid with 2 N HCl. An aliquot is taken to dryness, redissolved in 5 mM sodium acetate, and analyzed by reverse-phase liquid chromatography on a μBondapak C_{18} column. As little as 0.003% phytic acid in foods may be quantitated with a coefficient of variation of less than 5%. The high sensitivity and specificity, superior accuracy, reliability, and mechanical ease of the procedure and the lack of interference from high concentrations of protein and cations appear to make this method suitable for routine analyses of most food samples.

Much of the world's supply of food protein is derived from cereals and soybeans. With a continually expanding protein need, increasing emphasis is placed on developing the necessary food technology to use other plant proteins directly for human consumption. Concomitantly, in Western diets there is now a trend toward substituting meat with plant foods. Despite the caloric adequacy of these plant foods, they do have a lower mineral content and may interfere with the bioavailability of minerals. Reports in the literature suggest use of such foods may lead to human imbalances of Ca^{2+} (Mellanby, 1949), Mg^{2+} (Seelig, 1964), Fe^{3+} (Davies and Nightingale, 1975), and Zn^{2+} (Oberleas and Harland, 1981). Well-documented examples include Zn^{2+} deficiencies in some Iranian tribes (Reinhold, 1972) and in low-income preschool children in the United States (Hambidge et al., 1976).

The putative antinutritional agent present in plant foods that lowers the mineral bioavailability is phytic acid [*myo*-insoitol 1,2,3,5/4,6-hexakis(dihydrogen phosphate)]. It constitutes 1–6% by weight of most nutritionally important legume, cereal, and oilseeds (Cheryan, 1980), where it serves as a store of phosphate that becomes available during germination (Hall and Hodges, 1966). It forms insoluble complexes with di- and trivalent cations at neutral pH (Vohra et al., 1965), potentially rendering these minerals unavailable for intestinal absorption. Several recent review articles discuss the chemistry and nutritional implications of phytic acid (Maga, 1982; Cheryan, 1980; Erdman, 1979; O'Dell, 1979).

Most commonly used methods for the determination of phytic acid are modifications of the procedure developed by Heubner and Stadler (1914). Such methods involve the precipitation of an iron complex at low pH and the subsequent quantitation of phosphorus, iron, or inositol in the precipitate or analysis for residual iron in the supernatant (Oberleas, 1971). Valid criticism generated by numerous assumptions and uncertainties inherent in these methods has instigated the development of new analytical methods that are independent of the formation of an iron phytate complex. A high-performance liquid chromatographic method for the determination of phytic acid was recently reported (Graf and Dintzis, 1982) that combines an ion-exchange procedure (Harland and Oberleas, 1977) with HPLC on a μBondapak C_{18} column (Tangendjaja et al., 1980).

This paper describes a modification and application of our previously reported HPLC method for the determination of phytic acid in a variety of foods.

MATERIALS AND METHODS

Materials. Sodium phytate and boving serum albumin (BSA) were purchased from Sigma Chemical Co. All other chemicals were of reagent grade. Anion-exchange resin AG 1-X8 mesh 200–400 (Cl^- form) was obtained from Bio-Rad Laboratories. Disposable polypropylene minicolumns were purchased from Kontes Scientific Glassware. The HPLC equipment was from Waters Associates and consisted of the following modules: solvent delivery system, Model M-45; automated sample introduction system, Model 710B WISP; μBondapak C_{18} column (0.4 × 30 cm); absorbance detector, Model 440; differential refractometer, Model R401. UV absorption and refractive index were monitored on a Beckman two-channel potentiometric recorder; the refractive index peak areas were obtained through integration by a Modcomp Computer Model Classic 7870. All solvents were prepared with distilled, deionized water, filtered through a 0.45-μm Millipore filter, and degassed by sonication for 1 min. The liquid chromatography column was stored in 0.02% sodium azide.

HPLC Method for Phytic Acid Determination. After being lyophilized (Virtis lyophilizer), pulverized in a cyclone mill (Udy Analyzer Co., Boulder, CO) and defatted with petroleum ether for 4 h in a Soxhlet extractor,

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