Grain Sorghum Condensed Tannins. I. Isolation, Estimation, and Selective Adsorption by Starch[1]

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ABSTRACT

The biologically active condensed tannins and other polyphenolics were extracted from bird-resistant grain sorghum with methyl alcohol after preextraction of the whole grain with water-saturated butanol. The condensed tannins were separated from other polyphenolics with Sephadex LH-20; the polyphenolics were eluted with 95% ethyl alcohol and then the condensed tannins were eluted with 50% acetone. A nonbird-resistant sample gave no condensed tannins by the above procedure. The Phadebas α-amylase procedure used in conjunction with porcine pancreatic α-amylase provided a simple, rapid, and reliable method for determining the biologi-

cally active condensed tannins in both ground grain samples and extracted materials. The three varieties of bird-resistant grain sorghum differed little in terms of inhibition per unit weight of isolated condensed tannins. Condensed tannins isolated from three varieties of bird-resistant grain sorghum were adsorbed by starch. The amount of tannins bound by starch varied both with the source of tannins and with the starch species. Data suggested the presence of at least two fractions in condensed tannins of grain sorghum; an α-amylase inhibiting fraction, which was adsorbed on starch, and an inhibiting fraction, which was not adsorbed on starch.

Grain sorghum is the fifth most extensively grown crop in the world (Mabbayad 1974). In the United States, where grain sorghum is the third largest cereal crop harvested (Maxon et al 1973), its production is centered in areas where lack of moisture and high temperatures make production of other cereal crops unreliable (Bate-Smith and Rasper 1969, Neihaus and Schmidt 1970).

In many areas, grain sorghum is subject to depredation by birds, which accounts for annual losses of 18–75% (McMillian et al 1972); losses up to 100% in Louisiana (Tipton et al 1970) have been reported. In some areas, losses to birds are of more concern than losses to insects and disease. Sorghum that is high in tannins and has an open panicle structure deters attack by birds (Burns 1971, McMillian et al 1972, Neilhaus and Schmidt 1970, Tipton et al 1970). High tannin content also has been related to reduced germination in the head (Harris and Burns 1970).

High tannin content has resulted in several problems in the use of bird-resistant grain sorghums. These problems include lowered digestibility indices (Armstrong et al 1974, Chang and Fuller 1964, Connor et al 1969, Cummins 1971, Maxon et al 1973, McGinty 1968, Saba et al 1972, Schaffert et al 1975), possible carcinogenic effects (Morton 1970, Singleton and Kratzer 1973), lower palatability due to astringency (Bate-Smith and Rasper 1969), and off colors in milled products (Bate-Smith and Rasper 1969, Blessin et al 1963).

Many studies have determined the effects of bird-resistant hybrids on growth of meat animals (Armanious et al 1973, Armstrong et al 1974, Cummins 1971, Damron et al 1968, Davis 1973, Davis and Harbers 1974, Maxon et al 1973, McCollough 1970, 1973, Nelson and Cummins 1971, Potter and Fuller 1968, Rahman 1972, Rostagno et al 1973, Schaffert et al 1975, Vohra et al 1966) and on inhibition of a α-amylase activity (Davis 1973, Davis and Harbers 1974, Maxon et al 1973). Others have surveyed the polyphenolic materials in sorghum (Blessin et al 1963, Yasumatsu et al 1965) and developed methods for their separation and analysis (Damron et al 1968, Strumeyer and Malin 1975.)

Many chemical methods have been proposed for quantifying condensed tannins. Maxson and Rooney (1972) reviewed eight popular methods and found none to be fully satisfactory. Chemical methods for determining tannins generally suffer from the chemical similarity between tannins and other phenolics. The two groups are difficult to separate quantitatively but differ dramatically in

effect on biological systems. Many workers are now using quantitative determinations based on binding tannins to various proteins.

Most methods involve an enzyme, commonly α-amylase, and measure either substrate loss (Maxon et al 1973, Strumeyer and Malin 1975) or product formation (Daiber 1975) resulting from residual enzyme activity after exposure to tannin.

Davis (1973) and Davis and Harbers (1974) indicated that starch isolated by wet milling (from bird-resistant sorghum) is less susceptible to enzyme attack than was other similarly isolated sorghum starch. That suggests the possibility that starch adsorbs and retains condensed tannins in amounts detectable by their enzyme inhibitory effect. Strumeyer and Malin (1969) reported that starch is unable to prevent condensed tannins from inactiviting amylase.

Using a modification of the Phadebas α-amylase determination and UV absorbance, this study provides further information on the interaction of condensed tannins and starch.

MATERIALS AND METHODS

Our system for determining ezyme inhibition by polyphenolic materials of sorghum grain was a modification of the Phadebas Clinical α-amylase determination. The standard determination consisted of one Phadebas (Pharmacia Laboratories, Inc., Piscataway, NJ 08854) tablet plus 4.0 ml of water and either 0.25 ml of liquid (water or methyl alcohol) or appropriate weights of solid sample containing inhibitor. The materials were added to a 25-ml test tube, mixed, and brought to 30°C in a water bath. One milliliter of amylase was added, the contents were mixed again, and the mixture was incubated for exactly 10 min. Enzyme activity was stopped by adding 1.0 ml of 0.5 NaOH. Samples were diluted with 10 ml of water and filtered through Whatman 1 paper; absorbance was read at 620 nm on a Beckman DB-G spectrophotometer.

The enzyme used was twice crystallized porcine pancreatic α-amylase (Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63118). The enzyme was diluted with water (5 μl to 100 ml) immediately before each determination. Standard activity curves based on International Units (IU) per 100 ml were prepared for each lot of enzyme. Inhibition percentage was calculated by the following formula:

$$\frac{\text{Control activity (IU/1,000 ml)} - \text{sample activity (IU/1,000 ml)} \times 100}{\text{Control activity (IU/1,000 ml)}}$$

Sample standard curves and factors for converting IU to other common amylase measuring units are given in the literature that accompanies each order of Phadebas tablets. Throughout the investigation, all samples were run in at least duplicate.

Grain Sorghum Samples

Three hybrid varieties of bird-resistant sorghum grain (G-516 BR, G-459 BR, Funk BR 79) and one unidentified, nonbird-resis-

tant hybrid were grown in 1974 on land near Manhattan, KS. Sampling involved collecting several heads of grain, air drying, and then thrashing on a single head thrasher. After thrashing, the samples were stored in plastic bags at 4°C.

Scanning electron photomicrographs showed a testa layer in each of the three bird-resistant varieties, which agrees with the findings of Mabbayad (1974) and Dehlavi (1974) who associated bird resistance and tannin with the presence of a testa layer. The nonresistant variety did not have a testa layer.

Extraction and Purification of Condensed Tannins

Grain samples were cleaned and any adhering glumes manually removed, and the tannins isolated (Fig. 1). The whole grain was extracted with water-saturated butanol (for 200 g of grain, 600 ml of water-saturated butanol overnight followed by two 3-hr extractions with 400 ml of water-saturated butanol each), air dried, and ground through a 40-mesh screen on a Wiley mill. The 200 g of ground grain was subjected to five extractions, each with 400 ml of methanol; the first extraction was overnight and each subsequent extraction for 3–5 hr. The methanol solutions were combined, filtered (Whatman 1 paper), and vacuum concentrated to approximately 200 ml. The solution became cloudy during concentration and was centrifuged (1,500 × g for 15 min) to remove an oily fraction containing a small amount of finely divided particulate matter. The centrifugation was repeated after the final volume (200 ml) was reached to remove a small amount of a similar precipitate. The precipitates were discarded after it was determined that relatively large amounts produced only limited inhibition of α-amylase activity.

The methanol extract was placed on a column containing Sephadex LH 20 (15 × 4.5 cm) that had been equilibrated in 95% ethanol. The column was eluted with 95% ethanol until UV absorbance (280 nm) indicated that no material was eluting (approximately 2,000 ml). The ethanol fraction that contained other polyphenolics was determined to have essentially no enzyme inhibiting ability and was discarded. The column was then eluted with 50% aqueous acetone until absorbance at 420 nm (420 nm avoided the absorbance of the acetone solvent) of the effluent was nil (approximately 2,000 ml). The eluted material was exposed to a warm air current to remove the acetone, after which the remaining solution was freeze dried. The resulting fluffy brown powder (condensed tannins) was stored at room temperature until used.

Gel Filtration of Condensed Tannins

Material for gel filtration was dissolved (300 to 500 mg) in a small (usually 3 to 5 ml) amount of 50% aqueous acetone and separated by chromatography on a column of Sephadex LH 20 (18 × 3.5 cm) equilibrated in 50% aqueous acetone. Elution at approximately 2.5 ml/min was performed with the same solvent. Five-milliliter fractions were collected and absorbance was determined at 420 nm.

Condensed tannin adsorption on starch was determined by exposing 10 ml of 0.5 mg/ml condensed tannins solution to various amounts of starch (100 to 500 mg). After 30 min of exposure, the suspension was centrifuged to remove the starch and bound tannin. A 0.25-ml aliquot of the supernatant solution was added to the standard Phadebas determination to determine percentage of inhibition. An additional 1-ml aliquot was diluted with 10 ml of water and the absorbance determined at 280 nm to measure the amount of condensed tannin remaining in solution. Percentage of condensed tannin removed was calculated from the same formula used to calculate percentage of enzyme inhibition. The corn starch used was Argo brand from CPC International. Wheat starch was supplied by Commercial Solvents, Inc., Atchison, KS.

RESULTS AND DISCUSSION

Phadebas System

The Phadebas clinical α-amylase determination proved to be useful for evaluating condensed tannins in sorghum grain. Used in conjunction with porcine pancreatic α-amylase, it provides a simple, reliable method for determining amylase inhibition levels in both ground grain samples and extracted materials. Ground grain samples must be kept small (less than 50 mg) and appropriate

blanks run to compensate for colored materials extracted during incubation. Potential competition for enzyme between raw starch in the sample and the Phadebas substrate proved not to be a problem because adding 250 mg of waxy sorghum starch reduced Phadebas-determined enzyme activity less than 10%, and starch in amounts of 50 mg or less was undetectable.

Extracted materials must be presented to the Phadebas system in either water or methanol. Other solvents, such as ethanol and acetone, completely stopped enzyme activity.

Two difficulties with the Phadebas system are its fixed pH (optimized for human α-amylase at 7.0) and its varying susceptibility to enzyme from different sources. The first can be solved by adding buffer to adjust the pH to optimum for the enzyme in use (Barnes and Blakeney 1974), and the second by preparing new activity curves for each enzyme. In this study neither of the above was a problem because only porcine α-amylase was used and it has a pH optimum of 6.8. The Phadebas determination is most valuable because it provides a more rapid, simple, and direct measure of enzyme activity than does reducing power.

Enzyme Inhibition by Condensed Tannin Extracts

Condensed tannins were extracted from the three bird-resistant grain varieties following the scheme presented in Fig. 1. Completeness of extraction was determined by adding the dried grain residue from the methanol extraction to the standard Phadebas system. Additions of up to 300 mg of the residue produced no detectable enzyme inhibition. This was taken as evidence that the extraction scheme was effective in removing essentially all active condensed tannins.

The nonbird-resistant variety was extracted and purified by the same procedure used for the bird-resistant varieties. However, eluting the Sephadex column with 95% ethanol removed all UV-absorbing material; no further material was recovered by elution with 50% aqueous acetone.

All UV and visible absorption spectra, determined for condensed tannins from the three bird-resistant varieties, were essentially identical. Absorptivities ($a_{1cm}^{1\%}$) at 280 nm were determined for condensed tannin extracts of each grain variety and for gel filtration fractions. Absorptivity values ranged from 135 to 150 with no detectable pattern of change among varieties or fractions. An average value of 144 was calculated and used throughout the investigation. Strumeyer and Malin (1969) reported a value of 162 at 277 nm for condensed tannins extracted in a similar manner from Leoti sorghum. Somers (1966), who found a value of 116 for condensed tannins separated from wine, commented that his value was low and might indicate a higher average molecular weight for wine-condensed tannins than for other condensed tannins. These results

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                    WHOLE GRAIN
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                         | W-STD. BuOH
                         |
        RESIDUE ─────────┼───────── SOLUBLES
                         |
                    DRIED, GROUND
                         |
                    MeOH (5X)
                         |
    INSOLUBLE ───────────┼─── SOLUBLE
                              |
                         VACUUM CONCENTRATED
                              |
        INSOLUBLE ────────────┼───────── SOLUBLES
                                   |
                                   | LH-20
                                   |
        50% ACETONE ───────────────┴────────── 95% ETHANOL
      (CONDENSED TANNINS)                    (POLYPHENOLS, ETC.)
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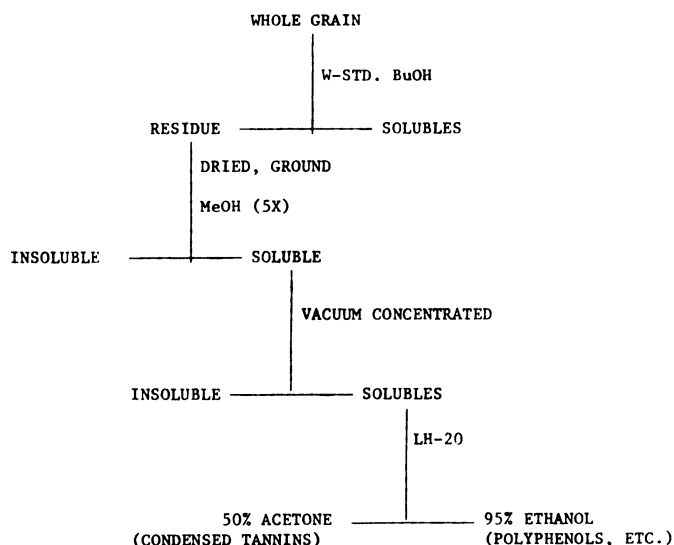
Fig. 1. Extraction and purification scheme for isolating condensed tannins from grain sorghum.

clearly show that use of UV absorbance for quantitation of condensed tannins requires specific absorptivity values for the material under consideration.

Raising the pH of condensed tannin solutions from pH 7 to 13 produced a 15-nm bathochromic shift and a marked increase in absorptivity. Strumeyer and Malin (1969) reported the same change in peak wavelength and noted an 80% increase in absorptivity.

A graph of percentage inhibition vs. condensed tannin concentration is presented in Fig. 2. We found little difference among the three varieties in terms of inhibition per unit weight of the isolated condensed tannins. Between 0.0115 and 0.014 mg/ml of condensed tannin, the change in inhibition was nearly linear and quite sensi-
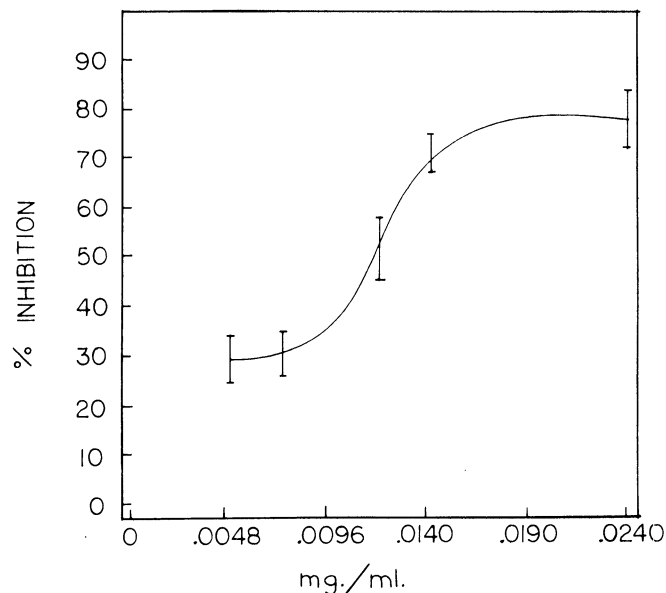

Fig. 2. Percent α-amylase inhibition vs concentration of condensed tannins (mg/ml). Standard deviation at 0.012 mg/ml = 4.3.
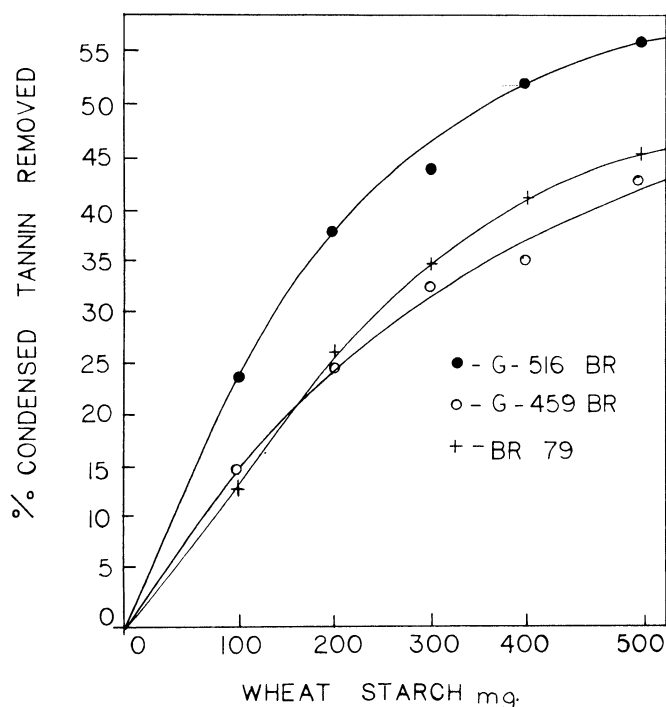
tive to condensed tannin concentration. At lower concentrations, results were unpredictable and at concentrations above the linear range, the determination became rather insensitive. These results are similar to those of Bate-Smith (1973) who used precipitation of hemoglobin to assay for tannins. His results, however, indicated linearity from a lower threshold of tannin concentration to complete precipitation. Our studies, which showed a loss of sensitivity in high ranges, indicated that enzyme bound to condensed tannin may not be completely inactivated until a large excess of condensed tannin is added. Daiber (1975) also mentioned that possibility in his work with condensed tannins in brewing.

Although Fig. 2 shows that percentage inhibition is sensitive to changes in condensed tannin concentration, the curve should not be considered a standard curve. The condensed tannins isolated by the scheme in this investigation are, at best, a crude fraction. No pure condensed tannin is available and most available evidence indicates that different condensed tannins may affect the enzyme system to different degrees. Therefore, we feel that results should be reported as percentage inhibition (what was actually measured) rather than on the amount of tannin that is based on a preparation of dubious purity.

Condensed tannin solutions allowed to sit overnight or longer formed a fine precipitate. Aged solutions lost inhibitory activity in most cases, but no pattern or rate of change was established. Possibly the precipitate is the result of polymerization leading to a molecular size that is insoluble. This may be similar to the proposed polymerization that Goldstein and Swain (1963) presented as a mechanism for loss of astringency in ripening fruits. They pointed out, however, that it is by no means certain that size alone determines the protein-binding ability of condensed tannins; molecular shape also may be a factor. Until the polymerization properties of condensed tannins are better understood, enzyme analysis and other measures of protein binding should be done on fresh solutions.

Adsorption of Condensed Tannins by Starch

Adding 10 ml of 0.5 mg/ml condensed tannin isolated from each sorghum variety to various amounts (100–500 mg) of wheat and corn starch and analyzing the supernatant solution for tannin, after centrifugation to remove the starch, gave a measure of the percentage of the original tannins bound by the starch (Fig. 3 and 4). The nonlinear relation between amount of starch and percentage tannin removed indicated that specific fractions of the condensed tannins
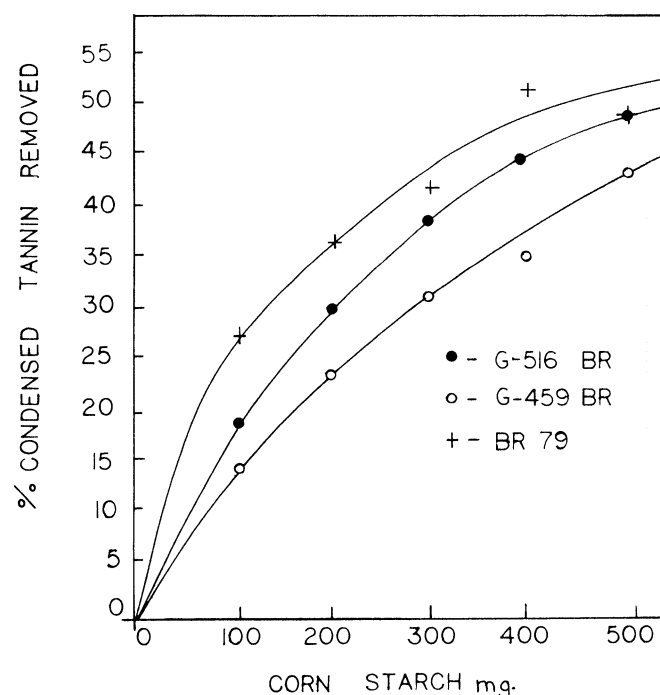

Fig. 3. Percent condensed tannin removed by certain amounts of wheat starch.

Fig. 4. Percent condensed tannin removed by certain amounts of corn starch.

were removed.

The general shape of the curves was similar for all varieties, suggesting that the maximum removal by starch would be 40–60% of the total tannins. Wheat starch removed more condensed tannins from G-516 BR and BR 79 than did corn starch. The tannins from G-459 were about equally adsorbed by both starches. Tannins from G-516 BR were more adsorbed on wheat starch than were those from BR 79. This relationship was reversed on corn starch; the tannins from G-459 BR were less adsorbed on corn starch than were those from either of the other two, but were nearly equal to those from BR 79 on wheat starch. Wheat starch might be expected to have more surface area per unit weight than corn starch because of the large number of small starch granules, which might explain the greater adsorption of condensed tannins on wheat starch. However, this would not explain the lack of increased adsorption of condensed tannin from G-459 BR on wheat starch. Also the different surface areas of wheat and corn starch cannot explain the reversal of the relative adsorptions of G-516 BR and BR 79 tannins on the two starches.

A sample of condensed tannins isolated from G-516 Br was passed through a column (8 × 2.5 cm) of wheat starch, which had been soaked overnight in water before being poured into the column. Approximately 40% of a 300-mg sample of tannins was recovered by extensive (1,000 ml) elution with water. The effluent was freeze dried and then separated by chromatography on Sephadex LH 20 (50% aqueous acetone). Figure 5 presents the elution patterns of both the original condensed tannin and the condensed tannins eluted from wheat starch. It is obvious that material was selectively removed in fractions eluting at about 50 and 75 ml. Enzyme inhibition (Table I) was determined on freeze-dried samples eluting to 45 to 60 ml (peak 1) and from 80 to 105 ml (peak 2).

These results indicate the presence of at least two fractions in the condensed tannins: an inhibiting fraction that was adsorbed on starch and an inhibiting fraction that was not adsorbed on starch. The evidence indicates that at least some of the condensed tannins were adsorbed selectively on starch and that the adsorption was a function of both the condensed tannin source and the starch source. Clearly the condensed tannins of grain sorghum are a complex multi-component group.

TABLE I
Percentage Inhibition (0.024 mg/ml) of Condensed Tannin Fraction of G-516 BR Eluted from Wheat Starch

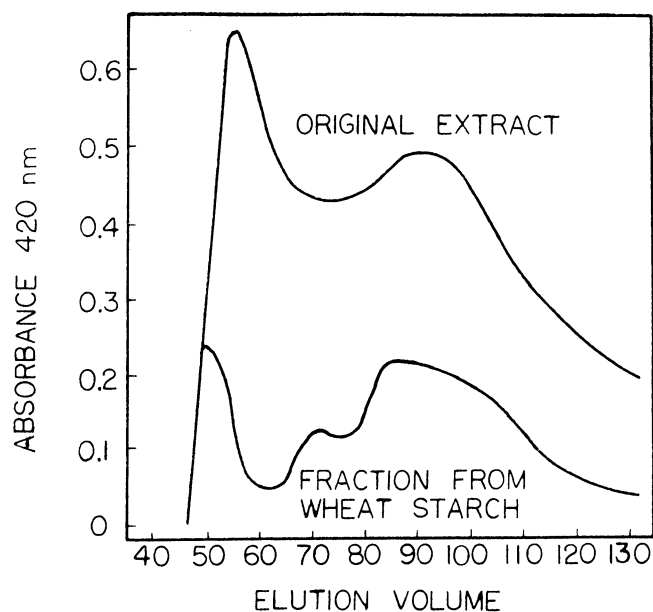| | Inhibition | |
Fraction	Original Solution %	Eluted from Starch %
Peak 1 (47–62 ml)	80.0	12.5
Peak 2 (80–108 ml)	51.4	15.5

Fig. 5. Elution profile of condensed tannins (G-516 BR) from Sephadex LH-20 with 50% acetone for the total extract and fraction not removed by wheat starch.

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