I. QUANTITATIVE DETERMINATION OF TANNIN IN GRAIN SORGHUM

II. PAPER CHROMATOGRAPHY OF SORGHUM TANNIN

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INTRODUCTION

Differences in palatability of the grain from different grain sorghum varieties have been attributed to relatively high tannin concentrations in the less palatable varieties. Although there is presumptive evidence that palatability and tannin content are related, there appears to be no direct experimental evidence for this relationship. Before this relationship can be investigated a satisfactory method must be established for the determination of tannin in grain sorghum seed. The low concentration of tannin normally present in sorghum grain precludes the use of the common methods for tannin measurement.

Colorimetric methods have been used frequently to measure low tannin concentrations. In this study, an investigation was made of several colorimetric methods, with emphasis on the spectrophotometric modification of two visual colorimetric methods which have been used specifically for tannin determinations in sorghum grain. A study was made of the variables common to both methods and between the two color reagents. In addition, the determination of tannin by several ultraviolet spectrophotometric methods was compared with the colorimetric methods. The purpose of these experiments was to select a method which would be most suitable for routine analysis of sorghum grain for tannin.

Since little is known about the nature of sorghum grain tannins, paper chromatographic studies of the tannins were carried out to determine the composition of this material. Most of the experiments were concerned with finding the solvent system or systems which would most effectively resolve the tannin components present in extracts of sorghum grain.

QUANTITATIVE DETERMINATION OF TANNIN IN GRAIN SORGHUM

REVIEW OF LITERATURE

Tannin is the generic name for a group of polyphenolic compounds which show certain characteristic physical and chemical properties and which are widely distributed in the plant kingdom. The most important chemical property of these compounds is their ability to form a stable complex with collagen in animal hide. This process, which is called tanning, is an important step in the manufacture of leather. Other characteristics include the precipitation of gelatin from solution, the formation of insoluble lead salts, the tendency to be readily oxidized, the formation of blue-black or green colors with ferric salts, the precipitation of many organic bases and an astringent taste (14).

Of the several classifications of tannins which have been proposed, the one most commonly accepted today classifies tannins into hydrolyzable and condensed tannins. The main distinction between these types of tannins is that the simple treatment of the hydrolyzable tannins with acid, alkali, or enzymes, splits them into sugars and recognizable phenolic carboxylic acids while the condensed tannins cannot be hydrolyzed into simpler components (24).

The information available as to the structure of tannins is limited. It must be pointed out that "tannin" from a given source is not a single compound, but a heterogeneous mixture of a number of related compounds. The structures of some components of myrobalans have been elucidated (20), however, and the structure of commercial tannic acid has also been proposed (24). Both of these tannins are classed as hydrolyzable tannins.

Structural information on condensed tannins is even more limited because a pure condensed tannin has never been obtained. It is known, however, that catechins are degradation products of some of these tannins, and from this information it has been postulated that some condensed tannins may be condensation products of catechins (24). According to Tsukunaga, et al. (22), the tannin found in sorghum seeds is of the condensed tannin class. No other work has been reported on the type or structure of tannins in sorghum.

A variety of methods has been employed for the quantitative determination of tannins. The method used depends on the type of tannin, its source and the amount present. Absorption of the tannins by hide-powder is used in the leather industry for estimating the tannin content from sources containing large amounts of tannin. The permanganate titration procedure of Loewenthal is the official quantitative method for the determination of tannins in tea, coffee and spices (2).

Two colorimetric methods have been reported for determination of tannin in sorghum grain. The first reported analysis for tannin from this source was made by Menaul (13) in 1923. He used an arsenic acid-sodium tungstate color reagent which produced a blue color with tannins upon the addition of sodium carbonate. Purified tannic acid was used as a standard for this determination. Tannin concentrations found ranged from 0.00 - 0.50%. Menaul recognized that this color reaction was not specific for tannins but stated that the tannins isolated with lead salt were the only compounds present which reacted with the color reagent. Snell and Snell (21) made reference to this method, reporting it as the arsenous acid-sodium tungstate reagent. When Barham and coworkers (3) tried the method described by Snell and Snell, their results were unsatisfactory; therefore,

they substituted a method using the Folin-Denis color reagent described by Valaer (23). This reagent was a phosphomolybdate-phosphotungstate solution originally investigated by Folin and Denis (6) who had applied it to a number of phenols and polyphenols. A characteristic blue color is given by the reaction of this reagent with both sorghum grain tannin and standard tannic acid. The values obtained by Barham, et al. (3) for the grain of several sorghum varieties ranged from 0.0030 - 0.1667%. This reagent is also nonspecific for tannins, and its use was based on the assumption that tannin was the only reagent positive material that was isolated using a procedure similar to that used by Menaul. These two methods are the only procedures which have been reported for determining the tannin content in sorghum grain.

Nitrous acid and sulfamic acid have been used for estimating catechol-type tannins. Ferric chloride, osmium tetroxide and ferrous tartrate have also been used to a limited extent (21).

Direct ultraviolet spectrophotometric analyses have been made on solutions of tannins in both the food and leather industries. Owades, et al. (15) used the absorption at 270mμ for the determination of tannins in hops and beer. Tannins isolated from the same source as the unknowns were used as reference standards. Roux (18) determined tannin content of black wattle extracts by the absorption at 280mμ. Since the absorption at this wavelength was not characteristic of any specific tannin, a 0.0084% solution of benzoic acid was used as an appropriate reference standard for comparison with 0.038% tannin solutions containing 0.1% of sodium sulfite as a stabilizer. In 1957, Roux (19) demonstrated that wattle polyphenols showed a much more intense peak at 202 - 203mμ than at 280mμ and, at this wavelength the concentration of the polyphenols followed Beer's Law with a high degree of concordance.

EXPERIMENTAL MATERIALS AND METHODS

The grain of five varieties of grain sorghum, ranging from white seeded to dark-brown seeded types was used for this investigation. These sorghums were grown by the Agronomy Department of Oklahoma State University, at Goodwell, Oklahoma, in 1956, and include Martin [1], Darset [2], Redlan [3], 4414 [4] and Wheatland [5]. The numbers following the varieties are sample numbers; subsequent reference to these samples will be by number only. The Beckman DU spectrophotometer was used for all photometric analyses.

The general procedure for extracting tannins from the grain involved a preliminary extraction with Skellysolve B to remove lipids followed by extraction of the tannin in 95% ethanol. The samples were finely ground in a Hobart grinder equipped with grain burrs and were thoroughly mixed prior to removal of sub-samples for extraction. All extractions were carried out in Soxhlet extractors. In the early part of the investigation large extractors (extractor capacity - 200 ml.) and 50 gram samples were used, while in later studies small extractors (extractor capacity - 50 ml.) and 10 gram samples were employed.

The tannin material was isolated from the crude extract by treatment with lead acetate which precipitates the tannin as lead tannate. The solution was centrifuged, the supernatant decanted and the residue of lead tannate treated with 5% sulfuric acid to redissolve the tannin and to precipitate the lead as lead sulfate. After the removal of the lead sulfate by centrifugation, the supernatant solution (denoted henceforth as the isolation solution) was used for tannin determination by reacting with

either the arsenic-tungstate or Folin-Denis reagents or by a direct ultraviolet spectrophotometric determination. Commercial tannic acid, extracted three times with ether to remove any gallic acid which might be present, was used as a reference standard.

In the determination of tannin using the arsenic-tungstate reagent, the isolation solution was treated first with the color reagent. A 20% sodium carbonate solution was then added, and the solution immediately diluted to volume. With the Folin-Denis reagent, the reagent was added to the isolation solution; five minutes later saturated sodium carbonate was added and the solution diluted to volume. Before the readings were made, except where otherwise noted, five minutes were allowed for color development of the arsenic-tungstate reagent and ten minutes for the Folin-Denis reagent.

A solution of purified tannic acid of known concentration was run simultaneously with and in the same manner as the sample. After establishing the linear relationship of concentration and optical density, the amount of tannin present in the sample was calculated directly from the optical densities of the standard and sample, in accordance with the Beer-Lambert equation.

EXPERIMENTAL RESULTS AND DISCUSSION

Preliminary Experiments

In the methods described by Menaul (13) and Barham, et al. (3), the estimation of the amount of tannin in sorghum grain was made by visual comparison of the colors of the standard and sample after treatment of the tannins with the color reagents. It was thought that a spectrophotometric measure of the absorption of the colored solution would give a more precise determination. A preliminary study was made on the absorption spectra of the colored solutions produced when tannic acid and sorghum tannin were treated with several color reagents. These included the Folin-Denis, arsenic-tungstate, arseno-tungstate and nitrous acid reagents. The arseno-tungstate reagent gave an absorption peak, but this peak shifted when different amounts of standard tannic acid were used. When standard tannic acid was treated with nitrous acid, the absorption spectrumdid not show a maximum. Of the four reagents tested only the Folin-Denis and the arsenic-tungstate reagents gave absorption maxima that did not change with concentration (1-10 mg.) and were the same for both sorghum tannin and standard tannic acid. The absorption maxima were at 650 and 675mμ for the Folin-Denis and arsenic-tungstate reagents, respectively. The absorption spectra of the standard and samples treated with these color reagents are illustrated in Figures 1 and 2. Since the Folin-Denis and arsenic-tungstate reagents showed the most promising results in these preliminary experiments, it was decided to investigate these two reagents further to ascertain whether one was more suitable than the other for the quantitative determination of tannin in grain sorghum.

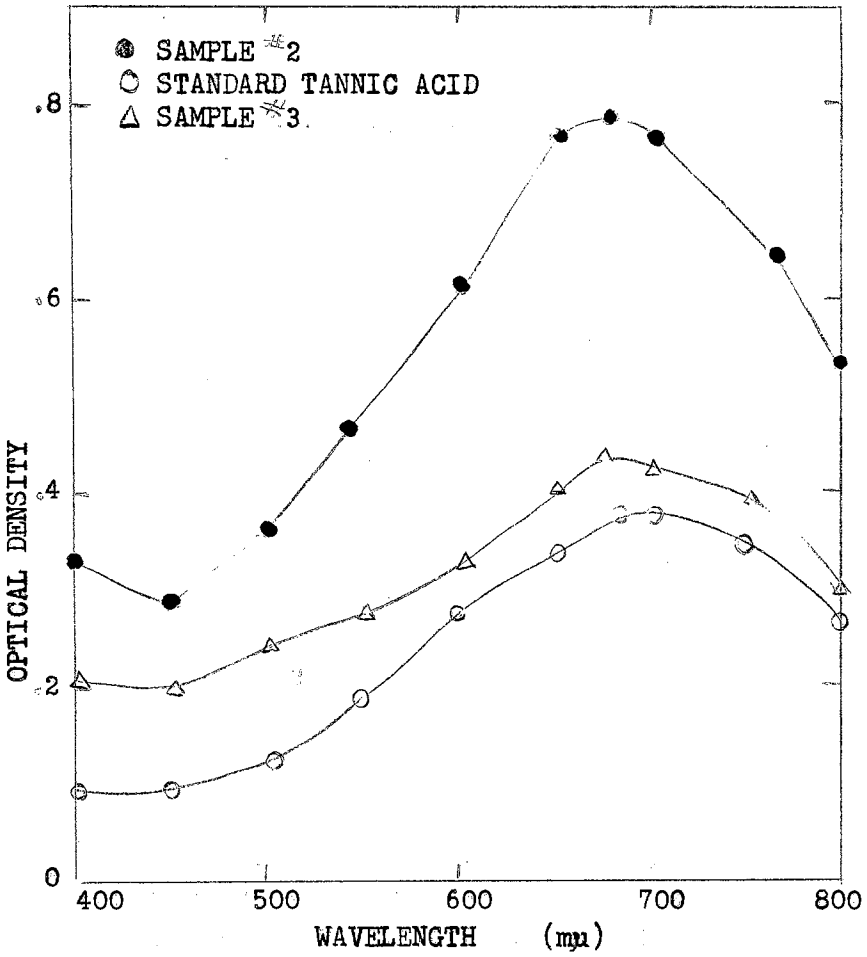


Figure 1

Absorption Spectra of the Colored Solution of Sorghum Tannin and Tannic Acid Treated with the Arsenic-tungstate Reagent

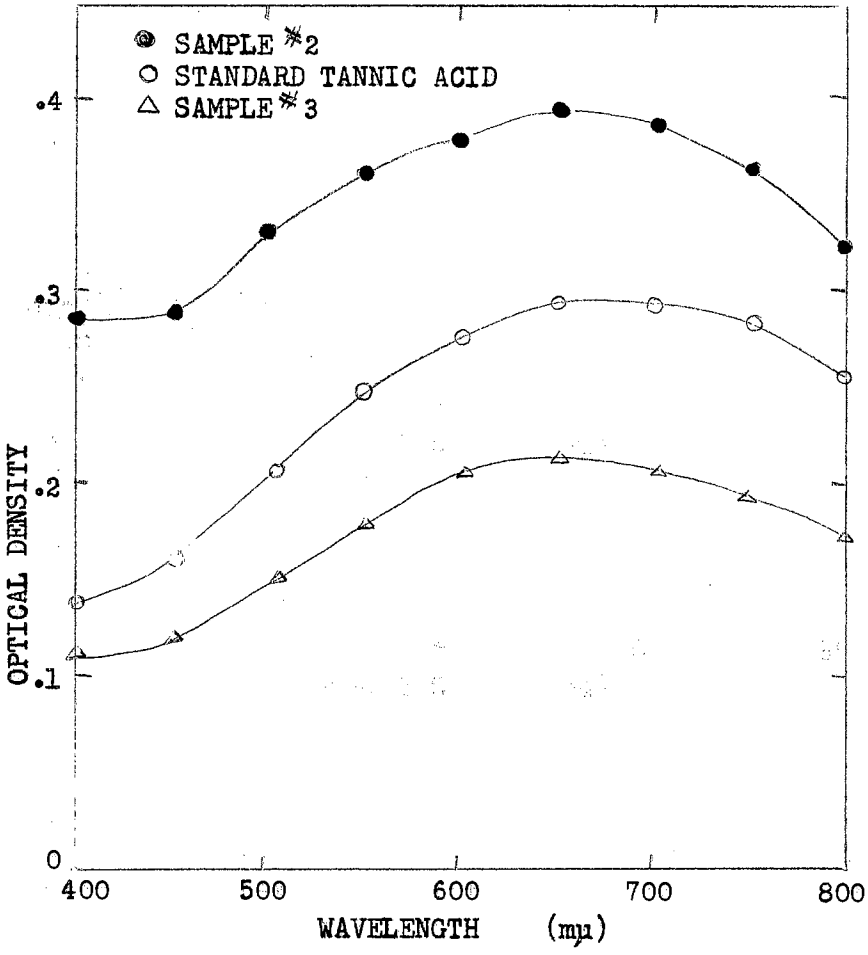


Figure 2

Absorption Spectra of the Colored Solution of Sorghum Tannin and Tannic Acid Treated with the Folin-Denis Reagent

Concentration Versus Optical Denisty

In order to calculate the amount of tannin directly from the standard, a linear relationship between concentration and optical density must hold true at the absorption maximum. This relationship was studied for both tannic acid and sorghum tannin using both color reagents and is graphically represented in Figures 3 and 4.

In the case of the arsenic-tungstate reagent the highest concentration for which Beer's Law holds true was 1.3 mg. for the standard and 0.6 mg. (apparent concentration) for Sample #2. The maximum concentration limit for the Folin-Denis reagent was 0.5 - 0.6 mg. for both standard and Sample #2. It was necessary to represent the tannin content of the samples as "apparent" concentration as determined by each reagent because the actual value for the tannin content was not known.

Procedure Studies

A. Extraction Procedure

It was observed in early experiments that there was a marked difference in the tannin content of different extracts of the same sample, as determined by the same color reagent. In these experiments the large extractors were used. The sample (50 grams) was first extracted with Skellysolve B for 20 to 24 hours and then with 95% ethanol for the same period of time. The data in Table I illustrate the variation between extracts as determined by the Folin-Denis color reagent.

It is obvious that the amount of tannin extracted from a given sample was not the same for all three sets of extractions. The large differences in the amount of tannin extracted for a given sample may be partially accounted for by variations in the amount of heat supplied to the

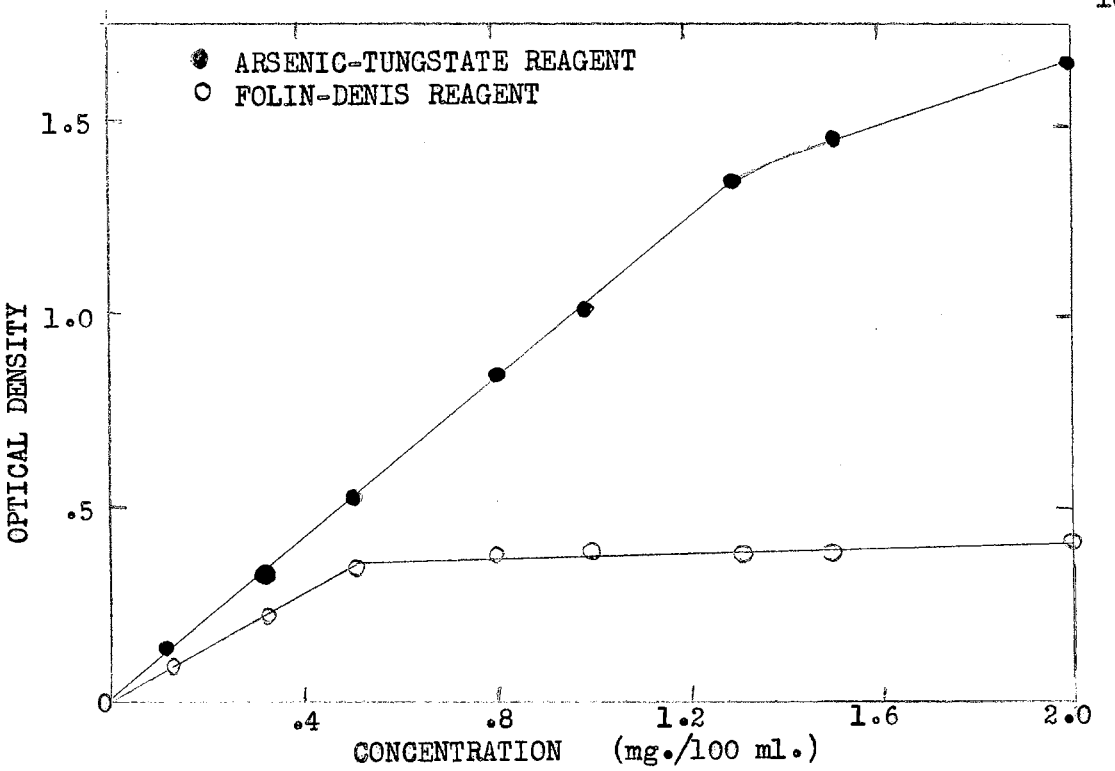


Figure 3

Concentration Versus Optical Density for Standard Tannic Acid

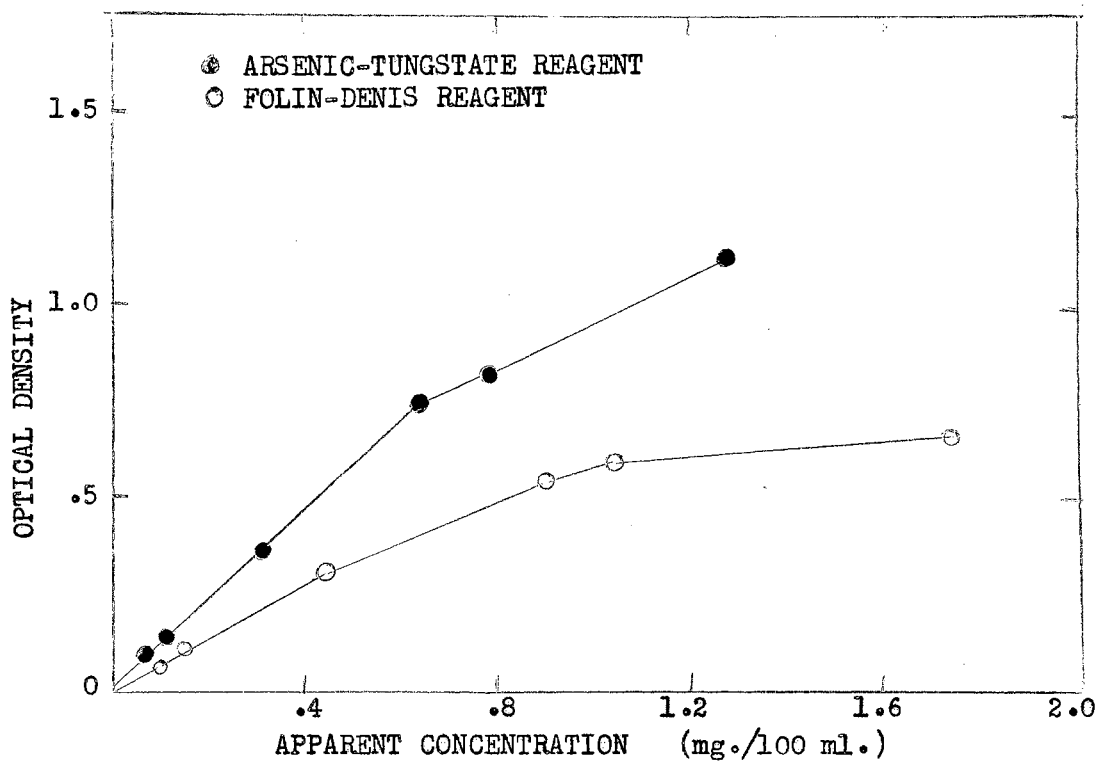


Figure 4

Concentration Versus Optical Density for Sample #2

individual extractors. The heat control for each unit could not be accurately regulated, and in some of the extractors the amount of heat supplied to the extractor was reduced by a space between the heating unit and the flask. The variation in the amounts of tannin extracted could probably have been reduced with these larger extractors by extracting for a longer time. The smaller extractors were used for the remainder of the investigation because an appreciably longer extraction time would not be desirable for routine analyses. With these smaller units the heat was more accurately controlled and there were more extraction cycles per unit of time than with the larger Soxhlets.

TABLE I

REPRESENTATIVE DATA SHOWING VARIATION AMONG EXTRACTIONS USING LARGE EXTRACTORS

Sample #	Percentage of Tannin in the Samples[1]		
	Ext.#1	Ext.#2	Ext.#3
1	0.0342	0.0287	0.0274
2	0.3350	0.1279	0.1677
3	0.0643	0.0530	0.0514
4	0.0131	0.0091	0.0109
5	0.0307	0.0190	0.0262

[1]Determined by the Folin-Denis reagent.

When 10 gram samples were extracted for 20 - 24 hours by each solvent (Skellysolve B and 95% ethanol) using the small extractors, there was much less variation in the tannin content of the alcoholic extracts than had been found with the large extractors. Since there was still some variation among extractions, it became necessary to establish the minimum time

for complete alcoholic extraction of the tannin from the grain. Ten grams of Sample #3 were extracted with Skellysolve B for about 18 hours (overnight), dried and extracted with 95% ethanol for different time intervals as shown in Table II.

TABLE II

THE EFFECT OF EXTRACTION TIME ON THE AMOUNT OF TANNIN EXTRACTED

Extraction Time (hours)	Percentage of Tannin in the Samples[1] Experiment #1	Experiment #2
2	0.03015	--
4	0.03496	0.03568
6	0.03472	0.03840
8	0.04165	0.04088
24	0.04582	0.04480
30	--	0.04496

[1]Determined by the arsenic-tungstate reagent.

In Experiment #1, the maximum time for extraction was 24 hours. The results were inconclusive; therefore, in order to ascertain whether or not any tannin was extracted beyond that 24 hours, a second experiment was run. The results shown indicate that extraction of tannin is essentially complete in 24 hours.

B. Procedure for the Isolation of Tannin

It had been noted in some early studies that there was sometimes a marked variability in results from the same extraction. Therefore, it seemed desirable to attempt to learn which step in the procedure was responsible for this variation. For all the tannin determinations made up

to this point in the investigation the following procedure had been used for the isolation of tannin from the crude extract. A 10-ml. aliquot of the extract was placed in a tapered 15 ml. centrifuge tube and 2 ml. of 10% lead acetate were added. The tube was heated in a water bath at 72 - 75°C until the precipitate was completely coagulated (about five minutes), then centrifuged. After the supernatant was discarded and the tube drained, five to ten drops of 5% sulfuric acid were added, depending on the amount of precipitate present. After thorough mixing, distilled water was added and the tube was centrifuged. The supernatant was saved, and the precipitate was washed with a small amount of distilled water. The wash water was combined with the supernatant for analysis.

In order to locate the source of the variability in this procedure an experiment was run in which duplicate isolations from the same extract as well as duplicate color developments on the same isolation solution were analyzed with the arsenic-tungstate reagent. The differences between duplicate isolations from the same extract were greater than the differences between duplicate color developments on the same isolation solution. The results of this experiment are presented in Table III. Because of the evident variability in the isolation procedure, a series of experiments was set up to find out if the variability in the isolation procedure could be reduced. In these experiments a control was run simultaneously with a set in which one of the steps had been altered.

One of the possible causes for variation was that some non-tannin, reagent-positive material might be adsorbed on the precipitate and carried over into the isolation solution. If this were the case, then washing the precipitate to remove non-tannin material might improve the reproducibility of the results. This was investigated by repeated washing of the lead

tannate precipitate with distilled water. It was found that the amount of tannin remaining in the precipitate decreased with each washing. When the washings were analyzed for tannin it was found that the combined washings increased in total tannin content with successive washings. These results indicated that some of the lead tannate was solubilized with each washing. The same type of results was observed when the lead tannate was washed with 10% lead acetate. After successive washing of the precipitate with 95% ethanol, however, there was no apparent decrease in tannin content, but variability was not decreased appreciably either. It was concluded from these results that washing the lead tannate would not effect any improvement in the procedure.

TABLE III

DATA ILLUSTRATING VARIABILITY IN THE ISOLATION AND COLOR DEVELOPMENT PROCEDURES

Sample #	Isolation	Percentage of Tannin in the Samples[1] Color Development		
		1	2	3
1	A	0.0231	0.0238	0.0241
	B	0.0252	0.0252	0.0265
2	A	0.1530	0.1510	0.1530
	B	0.1600	0.1620	0.1630
3	A	0.0430	0.0430	0.0404
	B	0.0512	0.0504	0.0524
5	A	0.0332	0.0326	0.0320
	B	0.0273	0.0269	0.0264

[1]Determined by the arsenic-tungstate reagent.

The hydrogen ion concentration of the solution of tannin prior to color development was considered as another possible source of variation in the procedure. Since the amount of acid added to redissolve the lead tannate was not accurately controlled, small differences in the amount of acid in the isolation solution could conceivably have an affect on the color development. To study this possible effect, aliquots were taken from the same isolation solution and adjusted to different pH values before treatment with the color reagent. The lowest pH represented the pH of the solution when ten drops of sulfuric acid were used to redissolve the tannin. This amount was a small excess of acid. When the minimum amount of acid needed to redissolve all the lead tannate was added the pH of the isolation solution of the samples ranged from 1.6 to 2.5. From the data presented in Table IV, it is apparent that an excess of acid did not affect the colorimetric reaction, but as the pH of the solution increased the results became erratic and were not the same for both reagents. There was more variation with the arsenic-tungstate reagent with change in pH than with the Folin-Denis reagent. With either reagent, however, it would appear that if enough acid is added to redissolve all the lead tannate, the addition of a small excess would not affect the final tannin determination.

The results from these experiments indicate that refinements at the particular points studied would not reduce the variability in the isolation procedure. It may be concluded that the isolation procedure originally used is the best method presently available for isolating the tannin from the crude extract.

TABLE IV

EFFECT OF pH OF THE TANNIN SOLUTION ON COLOR DEVELOPMENT

SAMPLE #1		SAMPLE #4	
pH	O.D. (arsenic-tungstate)	pH	O.D. (Folin-Denis)
1.7	0.165	1.6	0.108
3.2	0.165	3.4	0.108
4.2	0.118	4.3	0.100
5.5	0.058	5.5	0.109
7.0	0.146	7.0	0.102
9.0	0.105	9.0	0.088

Comparison of the Arsenic-tungstate Reagent and Folin-Denis Reagent

Briefly, the procedure for color development with the two reagents was as follows. For the arsenic-tungstate reagent 2 ml. of the reagent were added to the sample followed by the addition of 10 ml. of 20% sodium carbonate. The solution was then diluted to volume with distilled water and allowed to stand five minutes before the reading was made at 675mμ. In the case of the Folin-Denis reagent, 2 ml. of the reagent were added to the sample; after five minutes 5 ml. of saturated sodium carbonate were added and the solution brought up to volume with distilled water. The color was developed for ten minutes and then read at 650mμ. Distilled water was used for the blank in each case.

It was evident throughout this investigation that the two colorimetric reagents gave different results with the same sample, based on the same reference standard. In Table V, representative data illustrating this difference are presented. These values were obtained from aliquots of the same extract, thus eliminating this variable as a source for the difference.

For four of the samples, the Folin-Denis reagent gave tannin values ranging from 34 - 45% higher than the arsenic-tungstate reagent. For Sample #4, it was 23% higher.

TABLE V

PERCENTAGE OF TANNIN AS DETERMINED BY THE FOLIN-DENIS AND ARSENIC-TUNGSTATE METHODS

Sample #	Percentage of Tannin in the Samples	
	Folin-Denis	Arsenic-tungstate
1	0.0279	0.0199
2	0.1218	0.0912
3	0.0493	0.0344
4	0.0095	0.0077
5	0.0182	0.0125

The effect of time on color development was different for the two reagents. As shown in the upper graph in Figure 5, the optical densities of the colored solutions produced when the standard and sample were treated with the arsenic-tungstate reagent decreased rapidly with time. Since the optical density for the standard decreased at a slower rate than that for the sample, the apparent concentration decreased with time. This is illustrated in the lower graph in Figure 5. With the Folin-Denis reagent the optical densities for both standard and sample increased slightly for the first thirty minutes and then leveled off. Since this effect was similar for both standard and sample the apparent concentration remained constant over the three hour period for the Folin-Denis reagent. Before the first readings were made, five and ten minutes were allowed for color development with the arsenic-tungstate and Folin-Denis reagents,

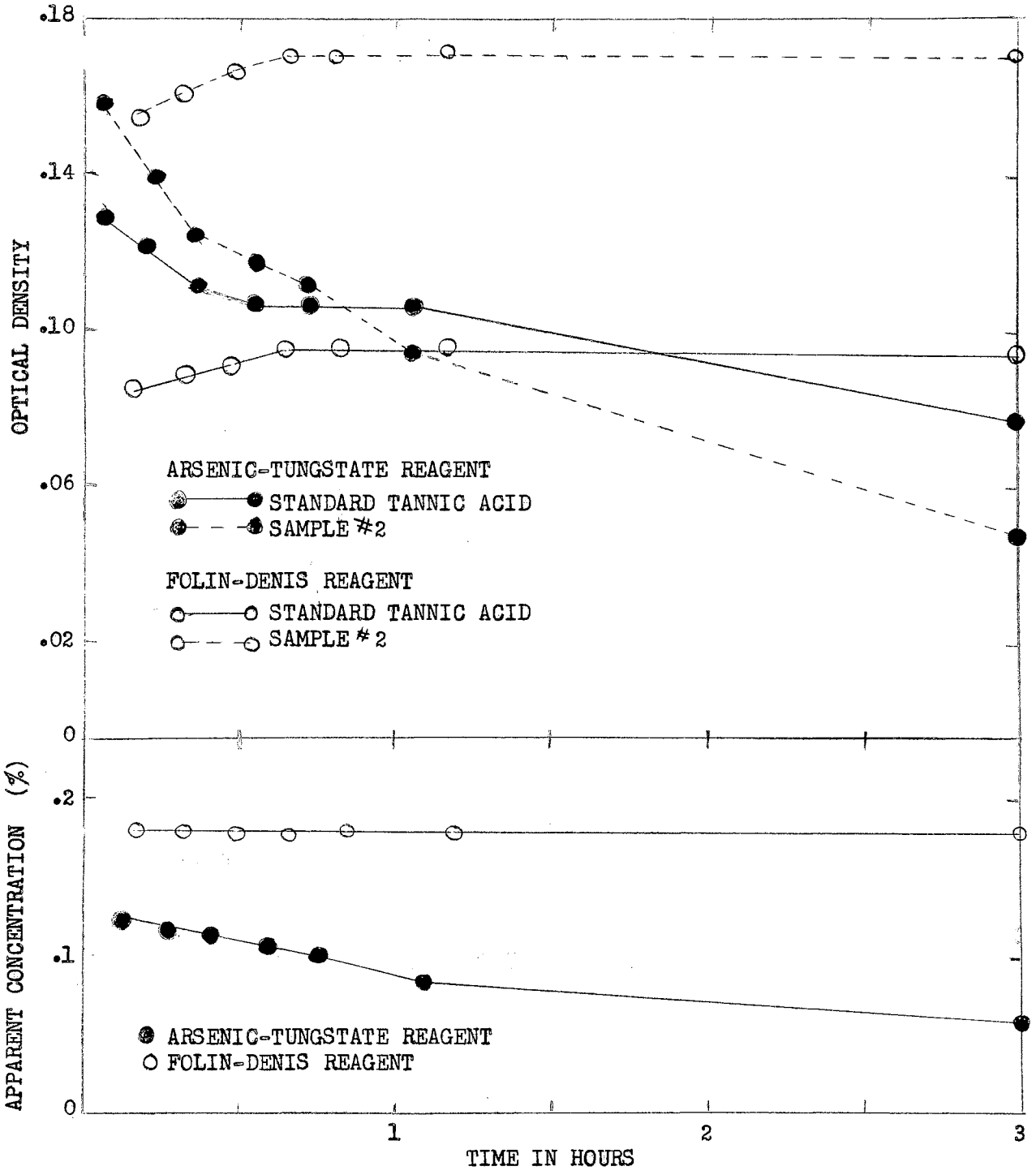


Figure 5

The Effect of Time on the Optical Density and Apparent Tannin Concentration

respectively. Since time between color development and reading is an important factor with the arsenic-tungstate reagent, it appears that there would be more possibility of variation with this reagent than with the Folin-Denis reagent. From this standpoint the Folin-Denis reagent would seem to be the better reagent for the determination of tannin in sorghum grain.

It was observed that with both color reagents a pronounced turbidity developed in the colored solution if a period of two minutes or more elapsed between the addition of sodium carbonate and the dilution to volume. Thus, with both reagents it was important to dilute to volume immediately after the addition of sodium carbonate.

From the results of these comparison experiments it was evident that there were marked differences between the arsenic-tungstate and Folin-Denis reagents. The reason for these differences is not known, but several possible causes can be advanced. It is known that sorghum tannin and tannic acid are not the same type of tannins; consequently, it might be expected that either or both color reagents would react somewhat differently with the standard than with the sample. This was found to be true for the arsenic-tungstate reagent where differences were observed between the standard and samples with respect to rate of fading and the relationship between concentration and optical density. For the Folin-Denis reagent, however, these differences between the standard and samples were not observed. Since neither reagent is specific for tannins there is also the possibility that some non-tannin material is isolated from the crude extract which reacts with the Folin-Denis reagent and not with the arsenic-tungstate reagent. This could possibly account for some of the differences in tannin content for the same sample as determined by the two color reagents.

Since there are these differences between the two reagents, a true evaluation with respect to the actual tannin content cannot be made until a pure tannin from grain sorghum is available.

Ultraviolet Spectrophotometric Methods

During the course of the investigation of the colorimetric methods, an ultraviolet spectrophotometric method for the determination of tannins in hops and beer was published (15). It was thought that this method might be applicable for the determination of tannin in sorghum grain; therefore, the method was investigated.

Ultraviolet absorption spectra were run on the standard solution and on the extracts and isolated solutions of the sorghum tannin. The maximum absorption for tannic acid was at 276 - 278mμ while the maxima ranged from 282 - 292mμ for the five grain sorghum samples. The absorption spectra of the extract and isolated solution were the same for each sample. The spectra of the standard and two of the samples are shown in Figure 6. Despite the fact that the standard and samples did not have their maxima at the same wavelength, for the purpose of comparison with the colorimetric methods, it was decided to estimate the amount of tannin in grain sorghum by measuring the absorption of the tannin at 278mμ, the maximum for tannic acid. Both sorghum extracts and isolated material were analyzed for tannin content and compared with the values determined by the colorimetric procedures. The results of these experiments are recorded in Table VI.

It is apparent from the data presented in this table that there was more material present in the extract which absorbed at 278mμ than there was in the solution of tannin isolated from the extract. These data also show that the amount of material measured as tannin by the ultraviolet

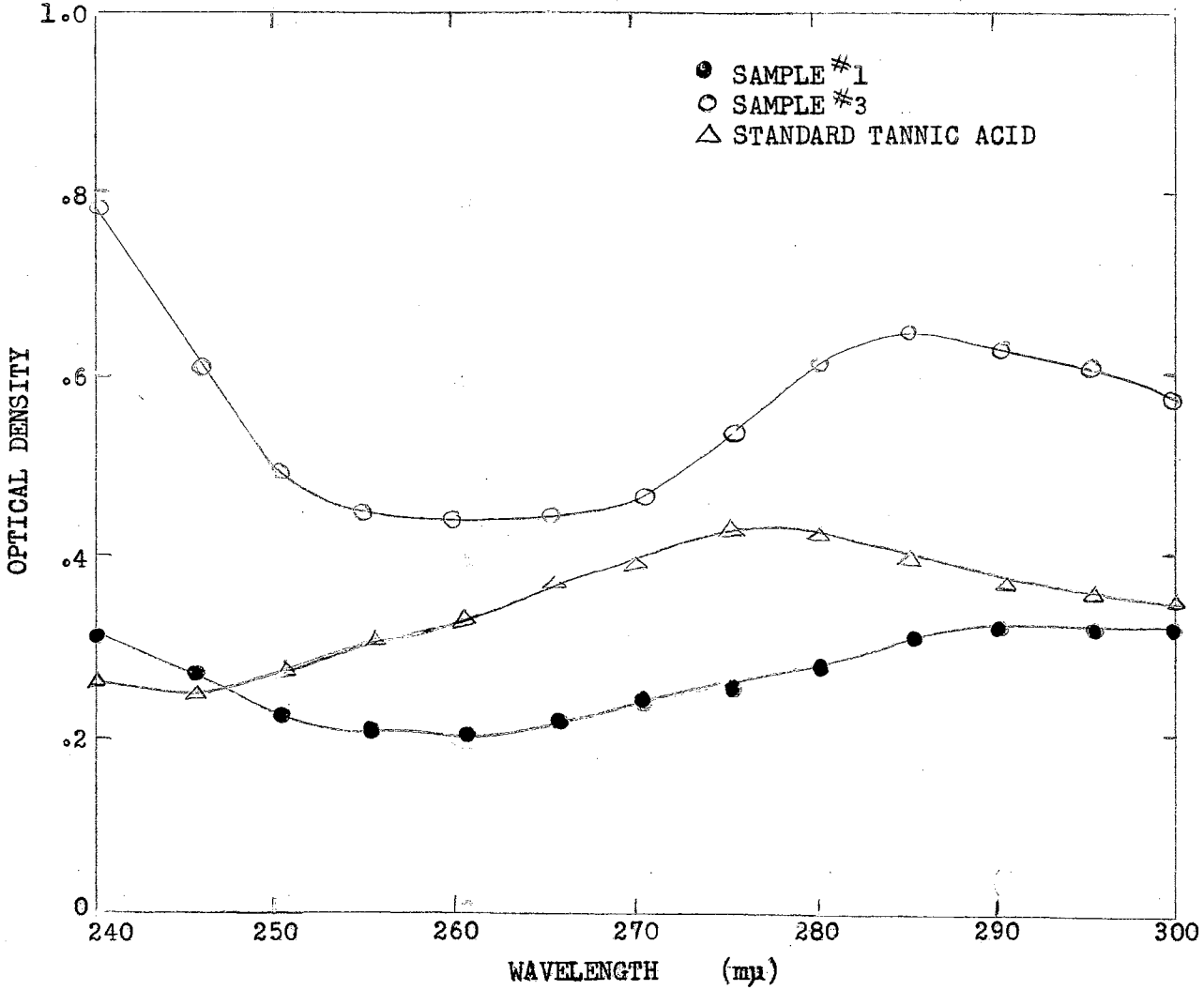


Figure 6

Ultraviolet Absorption Spectra of Aqueous Sorghum Tannin and Tannic Acid Solutions

method after the lead salt isolation was not the same as that determined by the colorimetric methods. The value for Sample #2 was lower than that for either color reagent while Samples #1 and #5 gave values between those determined by the two colorimetric methods. The values for Sample #3 and #4 were much higher than those obtained by the colorimetric methods.

TABLE VI

PERCENTAGE OF TANNIN AS DETERMINED BY AN ULTRAVIOLET METHOD (278mμ) AND TWO COLORIMETRIC METHODS

Sample #	Percentage of Tannin in the Samples			
	Extract Method U.V.	Isolated Tannin Method U.V.	Isolated Tannin Method Arsenic-tungstate	Isolated Tannin Method Folin-Denis
1	0.1100	0.0402	0.0294	0.0489
2	0.2060	0.1319	0.1550	0.2382
3	0.1603	0.0731	0.0442	0.0521
4	0.1070	0.0239	0.0108	0.0143
5	0.0926	0.0405	0.0246	0.0549

It was thought that a method of Roux (19) in which he determined the amount of tannin in wattle extracts by the absorption at 202mμ might be applicable for the determination of tannin in sorghum grain. Absorption spectra measurements between 200 and 240mμ were made for both tannic acid and sorghum tannin (extract and isolated tannin). Tannic acid gave an absorption maximum at 210mμ while the sorghum tannin did not give a maximum in this region of the spectrum. From the optical densities of the standard and samples measured at 210mμ the tannin concentrations were calculated. The results of this experiment are recorded in Table VII.

TABLE VII

PERCENTAGE OF TANNIN AS DETERMINED BY AN ULTRAVIOLET METHOD (210mμ)

Sample #	Percentage of Tannin in the Samples Extract	Isolated
1	0.172	0.0537
2	0.426	0.2120
3	0.352	0.0614
4	0.223	0.0262
5	0.162	0.0703

Except for Sample #3, the tannin content for the isolated material was higher than that recorded for the preceding ultraviolet method. These results were also generally higher than those measured by the colorimetric methods.

Another spectrophotometric method utilizing ultraviolet absorption at 280mμ and employing benzoic acid as a reference standard (18) was found to give exceedingly high results. Because of these extremely high results, this method was not investigated further.

A Statistical Comparison of the Two Colorimetric Methods

It became imperative at this point in the investigation to find out which of the color reagents, the Folin-Denis or arsenic-tungstate, would be the better for routine analyses. Since it is not known whether either reagent measures the true tannin content and since both reagents measure relative amounts of tannin it appeared that the only real basis for choice would be the precision obtainable. Therefore, a statistically designed experiment was set up so that information could be obtained that would

aid in making a choice. It was important to find out which method would be more precise when run on a routine basis. It was also of interest to know if the values obtained by the two colorimetric reagents were statistically different. Finally, it seemed desirable to evaluate the effects of different variables in the more precise method, and to learn which combinations of extractions, isolations and color developments, applicable to routine analyses, would give the smallest standard error of the mean for a variety. From this information it would be possible to obtain an estimate of the magnitude of differences (L.S.D.) which could be detected between any two varieties by the method selected for routine analysis.

The experiment designed to answer these questions was set up as follows. Two extracts of Samples #1, #2, and #3 were made according to the procedure described in the section on Extraction Studies. From each extraction, three 10-ml. aliquots were taken and the tannin isolated by the procedure described in the Isolation Studies. Instead of using the entire isolation solution for treatment with color reagents, however, each isolation solution was made up to 100 ml. and six aliquots from each isolation solution were taken for color development. Three of these aliquots were treated with the arsenic-tungstate reagent and the other three with the Folin-Denis reagent. After treatment with the color reagents the colored solutions were diluted to 25 ml. instead of 100 ml. in order to keep the optical density readings in the range normally encountered. This change necessitated a four fold dilution of the color reagents and carbonate solution. With these exceptions the procedure for the color development was the same as that outlined earlier.

The data obtained from this experiment were statistically analyzed. To determine which method gave the least amount of variation, the variances within each color reagent were calculated. The variance within the Folin-

Denis reagent was 3.236×10^{-6} while within the arsenic-tungstate reagent it was 1.1533×10^{-5}. An F test was made to test the homogeneity of these variances. The larger mean square (Folin-Denis) was divided by the smaller mean square (arsenic-tungstate). The value for F was 3.564 which is significant at the 1% confidence level. These results show that the color development with the arsenic-tungstate reagent has significantly smaller variability than that with the Folin-Denis reagent, and suggests that smaller differences between varieties could be detected with the method employing arsenic-tungstate reagent. Since the variances of the two reagents were significantly different, a 5% confidence interval for each mean was computed. It was found that the two intervals did not overlap and it was therefore concluded that the means of the two methods were statistically different. It is not known which method is biased with respect to the true amount of tannin present but the statistical evidence points out the more precise method.

After the procedure employing arsenic-tungstate had been established as the least variable method, an analysis of variance was made of data obtained by this method. Table VIII shows the analysis of variance and the calculated values for the parameters of variance. The results of the F test indicate that there were significant differences among isolations within extracts in each variety, the differences between extracts within varieties were significant and finally, there were significant differences among varieties.

Since the difference between varieties was the difference of interest, the following equation was used to calculate the variance of a varietal mean for a given combination of extractions, isolations and color determinations:

TABLE VIII

A. ANALYSIS OF VARIANCE FOR THE ARSENIC-TUNGSTATE REAGENT

Source of Variation	d.f.	Sum of Squares	Mean Square	F	Estimated Mean Square
Total	53	0.2071202999			
Between Varieties	2	0.2060627084	0.1030313542	692**	$\sigma_A^2 + 3\sigma_P^2 + 9\sigma_E^2 + 18\sigma_V^2$
Between Ext. in Varieties	3	0.0004481542	0.0001493847	3.64*	$\sigma_A^2 + 3\sigma_P^2 + 9\sigma_E^2$
Between Isol. in Ext. in Varieties	12	0.0004929424	0.0000410785	12.7**	$\sigma_A^2 + 3\sigma_P^2$
Within A-T in Isol. in Ext. in Var.	36	0.0001164949	0.0000032360		σ_A^2

B. CALCULATED VALUES FOR THE PARAMETERS OF VARIANCE

Parameter	Value x 10^7
σ_A^2	32.360
σ_P^2	126.142
σ_E^2	120.340
σ_V^2	571,566.497

* significant at the 5% level

** significant at the 1% level

$$\sigma_{\bar{V}}^2 = \frac{\sigma_A^2}{npk} + \frac{\sigma_P^2}{pk} + \frac{\sigma_E^2}{k}$$

Where n = No. of color determinations, A
p = No. of isolations, P
k = No. of extracts, E

By substituting the estimated values of the variances in the above equation and simplifying it, the following equation is obtained.

$$\sigma_{\bar{V}}^2 \; 10^7 = \frac{32.360 + 126.142n + 120.340np}{npk}$$

From this equation the variance of the mean of a variety can be estimated for any combination of extractions, isolations and color determinations used.

The estimated variance of a varietal mean was calculated for several combinations of n, p and k that might be applicable for routine analyses. These values are presented in Table IX. For each of these variances the Least Significant Differences were computed using the following formula:

$$\bar{d} = \sqrt{2}\, s_{\bar{V}}\, t$$

For example, in the combination of n = 2, k = 2, p = 2, a difference of 0.0103% in tannin content is significant at the 95% confidence level.

Least Significant Differences were calculated for a previous experiment in which the tannin content for all five samples was determined by the arsenic-tungstate reagent using three extractions, one isolation and two color determinations. The average values for the five samples and the differences between samples are given in Table X. Except for the difference between Sample #1 and #5, the difference between any two samples was larger than the Least Significant Difference (5% level). In this experiment, when n = 2, p = 1, k = 3, only Samples #1 and #5 were not significantly different in tannin content while, if an experiment were run in which the combination

TABLE IX

ESTIMATION OF THE VARIANCE OF A VARIETAL MEAN FOR A GIVEN COMBINATION OF n, p and k

Combination of n	p	k	$s_{\bar{V}}^2 \times 10^7$	Least Significant Difference 5% level	1% level
1	1	2	139.421	0.0665	0.3330
1	2	2	99.796	0.0143	0.0205
2	2	2	95.751	0.0103	0.0153
2	1	3	87.557	0.0107	0.0168
2	2	3	63.834	0.0079	0.0112

TABLE X

TEST OF SIGNIFICANCE BETWEEN THE FIVE SAMPLES

$\bar{d}$ (n=2, p=1, k=3) = 0.0107% tannin (5% level) 0.0168% (1% level)

Sample #	$\bar{v}$ (A-T)	Differences $\bar{v}$-0.0125	$\bar{v}$-0.0276	$\bar{v}$-0.0295	$\bar{v}$-0.0465
2	0.1550	0.1425**	0.1274**	0.1255**	0.1085**
3	0.0465	0.0340**	0.0189**	0.0170**	
5	0.0295	0.0170**	0.0019		
1	0.0276	0.0151*			
4	0.0125				

* significant at 5% level

** significant at 1% level

was n = 1, p = 1, and k = 2, Samples #1, #3, #4 and #5 would not be statistically differentiated (5% level) if the same varietal means (Table X) were obtained using this combination.

Recommended Procedure for Routine Determination of Tannin in Grain Sorghum

In selecting the proper procedure for routine analyses, several factors must be considered. The amount of grain available is important. This factor would be important for the determination of the tannin content of breeding material since the amount of material available would be limited to small quantities. The fact that the extraction step is the most time consuming step must also be considered. Other factors that enter into this consideration are the precision of the method used and the magnitude of difference in tannin content that would be important to the investigator. In the method to be described a difference of about 0.01% or more can be detected. It seems probable that smaller differences in tannin content between varieties would not be significant with respect to differences in palatability. This assumption cannot be verified, however, until tannin analyses and palatability studies are made on duplicate sorghum grain samples.

On the basis of the factors considered above and the evidence presented earlier the following procedure is recommended for routine determinations of tannin in sorghum grain. Two 10-gram samples of thoroughly mixed ground grain are weighed out into extraction thimbles. The samples are extracted first with Skellysolve B overnight, dried and then extracted with 95% ethanol for 24 hours, using the small Soxhlet extractors (extractor capacity - 50 ml.). The extracts are then cooled and stored in the

refrigerator for 48 hours, filtered and the filtrate made up to 100 ml. Duplicate 10-ml. aliquots are transferred to 15-ml. tapered centrifuge tubes and 2 ml. of 10% lead acetate are added to each aliquot. The tubes are then heated in a water bath at 72 - 75°C until all the lead tannate is coagulated (about five minutes). Then the precipitates are centrifuged, the supernatants discarded and the tubes drained for several minutes. Five to ten drops of 5% sulfuric acid (depending on the amount of precipitate) are added to each lead tannate precipitate and the suspension is thoroughly mixed to dissolve the tannate. The volume is then made up to about 10 ml. with distilled water and the tubes are centrifuged. Each supernatant solution is placed into a separate 100-ml. volumetric flask, and the precipitate is washed with 2 - 3 ml. of distilled water, centrifuged, and the wash water combined with the supernatant. The volume is made up to 100 ml. with distilled water.

Depending on the relative amounts of tannin in the samples, duplicate 5-ml. or 10-ml. aliquots of each isolation solution are placed in 25 ml. volumetric flasks. Two milliliters of a 1 - 4 diluted arsenic-tungstate reagent (see Appendix, Part A) are added to each flask followed by the addition of 10 ml. of 5% sodium carbonate. The volume is made up to 25 ml. immediately and after five minutes the reading is made at 675mμ using the Beckman DU spectrophotometer. For each set of color determinations, 0.025 mg. of a standard purified tannic acid is run using the same color development procedure. The per cent tannin in the grain sorghum varieties is calculated from the following equation:

$$\% \text{ Tannin} = \frac{\text{O.D. of sample}}{\text{O.D. of standard}} \times 0.025 \times \text{dilution factor (20 or 10)} \times 0.1$$

SUMMARY

Of the four colorimetric methods initially investigated only two, the Folin-Denis and arsenic acid-sodium tungstate reagents, were found to be suitable for the determination of tannin sorghum grain. These two colorimetric methods were investigated further in an attempt to determine which method would be the more applicable for routine analyses.

The linear range of concentration versus optical density for the standard tannic acid was about twice as great with the arsenic-tungstate reagent as with the Folin-Denis reagent. The range of linearity for the sorghum tannin concentration was found to be about the same for both reagents.

The procedures common to both colorimetric reagents were studied. Extraction time of twenty-four hours was found to be the minimum time needed for complete alcoholic extraction of the tannin. The isolation procedure was investigated in an attempt to reduce the variability present in the procedure. The variability in the results was not reduced when certain steps in the procedure were modified. From this study it was concluded that the isolation procedure, as originally used, was the best one presently available.

The arsenic-tungstate reagent gave better precision when the two colorimetric methods were compared statistically. Other information obtained from the statistical analysis of these methods included the following: the percentages of tannin for the same sample as determined by the two methods were statistically different; the variance of a

varietal mean was estimated for the arsenic-tungstate reagent using a given combination of extractions, isolations and color determinations; the Least Significant Difference was calculated for several combinations of n, p and k that would be applicable for routine analysis.

Several ultraviolet methods were investigated as possible means for the determination of tannin in sorghum grain. It was found that the absorption spectra for the samples were not the same and were also different from the spectrum for tannic acid. Because of this fact the ultraviolet methods as used in this study were not satisfactory for determination of tannin in sorghum grain.

A recommended procedure for routine analyses is proposed which is based on the experimental results obtained in this investigation.

PAPER CHROMATOGRAPHY OF SORGHUM TANNIN

REVIEW OF LITERATURE

Paper partition chromatography was first employed by Consden, et al. (5) to separate amino acids. Their method was a modification of column partition chromatography originally reported by Martin and Synge (12). The general principle involved is that the substances to be separated are distributed between two liquid phases. The liquid stationary phase is supported on treated (5) or untreated paper. Many different modifications of Consden's technique have been devised for paper chromatographic work.

The use of paper chromatography for the investigation of tannins in sorghum grain has not been reported in the literature. There have been, however, a number of investigators who have used this technique to study the complexity of tannin extracts from a wide variety of other sources. It has been demonstrated by paper chromatography that the tannins from most plant sources are mixtures of a number of related components.

A number of solvent systems have been employed in attempts to resolve the tannins from different sources. Mixtures of water and various alcohols have been used extensively to show the complexity of many tannin extracts. Roux (16) stated that n- and iso-propyl alcohol:water (7:3); tert-butyl alcohol:water (7:3); water-saturated tert-amyl alcohol and sec-butyl alcohol mixtures gave excellent fractionation of wattle extracts. A butyl alcohol:acetic acid:water mixture (4:1:5) has been used in studies on a number of tannins (8,9,10,17,25). A phenol:acetic acid:water system has been adapted by several workers (1,9,16) for separating tannins. Other solvent systems which have also been used include methyl ethyl ketone:

water, meta-cresol:acetic acid:water, dilute hydrochloric or acetic acids and many others.

Two-dimensional chromatography has been used by several investigators to resolve tannin extracts into their components. Haddaway (7) used an adaptation of the circular filter paper technique to separate a mixture of different types of tannins.

Several color reagents have been used to locate and identify tannins on paper chromatograms. These include Tollen's reagent and freshly prepared bis-diazotized benzidine, both of which are very sensitive. Location of spots for some tannins has been possible by virtue of their flourescence under ultraviolet light (7,8,9,10,11,16,17).

EXPERIMENTAL MATERIALS AND METHODS

In preliminary experiments commercial quebracho tannin and commercial tannic acid were chromatographed in addition to the extracts and isolated tannins from the same varieties of grain sorghum used in Part I of this investigation. The isolation procedure described in the first part of this thesis was used to isolate the sorghum tannin from the crude extracts of all five samples. Because more concentrated samples were needed for the chromatographic experiments larger quantities of tannin were isolated. The supernatant from each sample was taken to dryness under partial vacuum. The tannin was then redissolved in a minimum amount of absolute methanol.

A variety of solvent systems was used in this part of the investigation. Alcohols, phenols, mineral acids and methyl ethyl ketone were some of the solvents used. A more extensive discussion of these systems is given under Experimental Results.

Whatman No. 1 filter paper strips, one inch in width, were employed for most of the chromatographic experiments. Paper strips, four inches in width, were used only for the experiment in which the components were eluted and rechromatographed. The length of the strip varied with the type of chromatographic chamber used.

Spray reagents that were used for the identification of the spots included the arsenic-tungstate reagent (applying color reagent first, followed by 20% sodium carbonate), ammoniacal silver nitrate, freshly prepared bis-diazotized benzidine, 1% aqueous ferric chloride and Folin-

Denis color reagent (applying color reagent first, followed by saturated sodium carbonate). The preparations of these spray reagents are given in the Appendix, PartsA and B.

Three types of chromatographic chambers were employed at different stages of the investigation: a large wooden chamber, glass chromatographic jars and milk bottles ($\frac{1}{2}$ gallon and quart sizes). Descending chromatography was employed with the larger chromatographic chambers with one exception, while ascending chromatography was used entirely with the milk bottles. A chromatographic jar employing ascending chromatography was used in the eluting experiment.

With the larger chambers, techniques for applying the material, equilibration and identification of spots on the chromatograms were adapted from techniques commonly used by other investigators. In the experiments using the milk bottles, the paper strip was attached by means of a cup hook screwed into the under side of a rubber stopper. When equilibration of the paper with solvent vapor was employed, the paper was loosely folded over and attached by a paper clip and allowed to equilibrate for a definite time. One end of the paper was then placed in the solvent at the bottom of the bottle and the chromatogram developed. It was then set aside to dry. Location of tannin spots was accomplished by dipping instead of spraying as used in earlier experiments.

EXPERIMENTAL RESULTS

Preliminary Experiments

A. Qualitative Tests

Several qualitative tests that have been used to identify tannins were run on the grain extract to help characterize the material. The sorghum tannin gave positive results for the following tests: [1] precipitation of gelatin from solution, [2] green color when treated with ferric chloride and [3] precipitation when treated with lead acetate. These tests all strongly indicated that the material tested was tannin. According to Nierenstein (14), most condensed tannins give a green color when treated with ferric chloride while hydrolyzable tannins give a blue-green color. A known condensed tannin, quebracho, also gave a green color while tannic acid, a known hydrolyzable tannin, gave a blue-black color. An intense red color typical of condensed tannins was produced when both sorghum and quebracho tannins were treated with several drops of concentrated sulfuric acid while no color was produced when tannic acid was treated in a similar manner. The results of treatment with ferric chloride and sulfuric acid support the conclusions of Tsukunaga, et al. (22) that sorghum tannin should be classed as a condensed tannin.

B. Technique Studies

Three of the five spray reagents used were found to be the most sensitive. These were the arsenic-tungstate, ammoniacalsilver nitrate and bis-diazotized benzidine reagents. The blue color produced by the reaction of the arsenic-tungstate reagent with tannins was the most stable

in that it did not fade for about a week, while the other two sensitive reagents faded or the paper darkened within twenty-four hours; therefore, this reagent was used for locating the tannin on the chromatograms in most of the subsequent experiments.

The arsenic-tungstate reagent was found to be sensitive to about 5μg. of tannin material. Ten to fifteen μg. produced a pronounced color so that the spots could be easily located; consequently, this amount of tannin was used on most of the chromatograms. To determine the volume of solution to be put on the chromatogram, total solids and tannin analyses were made. From these values, the correct volume was calculated.

Investigation of Solvent Systems

A. Survey of Solvent Systems

Since no previous paper chromatographic work on sorghum had been reported, selection of solvent systems to be tried was based on work reported on tannins from other sources. A summary of results with these and other solvent systems is given in Table XI. When the isolated sorghum tannin was chromatographed using these solvent systems three types of results were observed. With a few of the chromatograms there was very little movement from the origin. It was observed that a majority of the solvent systems caused the tannin to streak about one-fourth the distance from the origin to the solvent front, while with several systems there was evidence of even more pronounced streaking. It was apparent from these results that all the systems attempted failed to resolve the sorghum tannin into more than one component.

The results produced when tannic acid and quebracho tannin were chromatographed using the butyl alcohol:acetic acid:water (4:1:5) solvent

TABLE XI

SOLVENT SYSTEMS EMPLOYED AND THE RESULTS OBTAINED IN PRELIMINARY INVESTIGATION OF CHROMATOGRAPHY OF TANNINS

Solvent System	References	Tannin Chromatographed	Technique Used[1]	Conditions Used[2]	Results
1. n-butyl alcohol:AcOH[3]:water(4:1:5)	(4,8,9,25)	Quebracho	descending	E-1[3]	streaking from origin
		Tannic acid	"	"	spot followed by long streak
		Crude sorghum tannin	"	"	streaking from origin; no resolution
		Isolated sorghum tannin	ascending	N.E.[3]	"
2. wet-sec-butyl alcohol	(9,10,16,17)	"	descending	E-1	"
		"	ascending	N.E.	"
3. phenol:AcOH:water (3:50:sat.)	(1,8,16,17)	"	descending	E-1	"
		"	ascending	N.E.	"
		"	"	E.[3]	"
4. sat. tert-amyl alcohol	(8)	"	"	N.E.	"
5. tert-amyl alcohol:AcOH:water(50:2:48)	(8)	"	"	"	"
6. sec-butyl alcohol:AcOH:water(50:2:48)	-	"	"	"	"

TABLE XI (continued)

Solvent System	References	Tannin Chromatographed	Technique Used[1]	Conditions Used[2]	Results
7. tert-butyl alcohol:water (7:3)	(8)	Isolated sorghum tannin	ascending	N.E.	streaking from origin; no resolution
8. n-propyl alcohol:10% AcOH (7:3)	-	"	"	"	"
9. n-propyl alcohol:water(7:3)	(8)	"	"	"	"
10. iso-propyl alcohol:water (7:3)	(8)	"	"	"	"
11. 1-15% aqueous AcOH solutions	(25)	"	"	"	"
12. 2N HCl	(25)	"	"	"	"
13. 2N HCl:10% AcOH(1:1)	(25)	"	"	"	"
14. n-butyl alcohol sat. with 5% NH_4OH	(4)	"	"	"	"
15. m-cresol:AcOH:water(50:2:48)	(4)	"	"	"	"
16. benzene:AcOH:water(2:2:1)	(4)	"	"	"	"
17. ethyl ether:butyl alcohol: acetone (2:3:3)	(7)	"	"	"	"
18. n-butyl alcohol:AcOH:water (4:1:5) plus 10% ethylene glycol	(11)	"	"	"	"

TABLE XI (continued)

Solvent System	References	Tannin Chromatographed	Technique Used[1]	Conditions Used[2]	Results
19. n-butyl alcohol:pyridine (50:20)sat. with sat. NaCl	(11)	Isolated sorghum tannin	ascending	N.E.	streaking from origin; no resolution
20. ethyl acetate:AcOH:water 95% ethyl alcohol (3:1:3:0.5)	(11)	"	"	"	"
21. pyridine:n-butyl alcohol: water(4:6:3)	-	"	"	"	"
22. n-butyl alcohol:pyridine: sat. NaCl(4:6:3)	-	"	"	"	"
23. n-butyl alcohol:AcOH:water: ethyl alcohol(4:1:5:0.5)	-	"	"	"	"

[1]Ascending - milk bottles; Descending - large cabinets.

[2]All chromatograms were run at room temperature.

[3]Abbreviations: AcOH - acetic acid; E-1 - equilibration for one hour; N.E. - no equilibration; E. - overnight equilibration.

system were not in agreement with those obtained by the other investigators. No explanation for this disagreement can be made. Because the main interest in this investigation was concerned with sorghum tannin and not the other tannins, chromatographic experiments on quebracho and tannic acid were discontinued.

After testing several of the more common solvent systems it became apparent that a large number of different solvent systems would need to be investigated in order to find a system that might resolve the sorghum tannin. To accomodate a large number of chromatograms in a short period of time, milk bottles were used.

B. Methyl Ethyl Ketone Solvent Systems

When a water-saturated methyl ethyl ketone system was first used with descending chromatography, a single spot close to the origin was observed. Since this organic solvent was the only one out of the many systems attempted that had moved the tannin from the origin without streaking, extensive chromatograms, using other solvents in combination with the ketone, under several different conditions, were run in the milk bottles. Equilibration, non-equilibration, room temperature and refrigeration (5°C ∓ 2°C) were the different conditions studied. A summary of the different solvent systems and conditions used for chromatography of the sorghum tannin, and the results obtained, are given in Table XII.

Methyl ethyl ketone:water systems failed to resolve the tannin into more than one component. The same type of results were observed when a methyl ethyl ketone:acetic acid:water (50:2:50) system was run at room temperature without equilibration.

Evidence for more than one sorghum tannin component was first observed when the methyl ethyl ketone system contained a higher percentage

TABLE XII

CONDITIONS USED AND RESULTS OBTAINED IN CHROMATOGRAPHIC EXPERIMENTS WITH SORGHUM TANNIN[1] EMPLOYING OTHER SOLVENTS IN COMBINATION WITH METHYL ETHYL KETONE

Solvents Used With Methyl Ethyl Ketone	Conditions Used		Results
	Temperature[2]	Equilibration[3]	
1. water (sat.)	R.T.	no	streaking from origin; no resolution
	R.T.	yes	"
	Ref.	no	"
	Ref.	yes	"
2. water (5% and 10%)	Ref.	no	"
3. acetic acid:water (50:2:50)	R.T.	no	"
4. 5% acetic acid (sat.)	R.T.	no	"
	Ref.	no	two spots; R_f values of ~0.4 and 1.0
5. 10% acetic acid(5% and 10% by volume)	R.T.	no	streaking from origin; no resolution
6. 10% acetic acid (sat.)	Ref.	no	two spots; R_f values of ~0.8 and 1.0
	Ref.	yes	"
	R.T.	yes	two spots; R_f values of ~0.8 and 1.0 but more spread out and more trailing

TABLE XII (continued)

Solvents Used With Methyl Ethyl Ketone	Conditions Used		Results
	Temperature[2]	Equilibration[3]	
6. 10% acetic acid (sat.)	R.T.	no	streak near solvent front; no resolution
7. 10% acetic acid (1:1)	Ref.	no	two spots; R_f values of ~0.8 and 1.0
	Ref.	yes	"
	R.T.	no	one spot; R_f value of 1.0
8. 15% acetic acid (1:1)	Ref.	no	two spots; R_f values of ~0.8 and 1.0
	Ref.	yes	"
	R.T.	no	one spot; R_f value of 1.0
9. 5% NH_4OH	Ref.	no	streaking from origin; no resolution
10. n-butyl alcohol:10% acetic acid	Ref.	no	"
11. sec-butyl alcohol:10% acetic acid	Ref.	no	one spot; R_f value of 1.0
12. tert-butyl alcohol:10% acetic acid	Ref.	no	streaking from origin to solvent front
13. pyridine:sat. NaCl	Ref.	no	streaking from origin; no resolution

[1]The isolated sorghum tannin from Sample #2 was used entirely in these experiments.
[2]Abbreviations: R.T. - room temperature; Ref. - refrigeration.
[3]Overnight equilibration.

of acetic acid and the temperature was reduced. From the chromatograms using the ketone with 5, 10 or 15% acetic acid it was obvious that there was better resolution when they were run under refrigeration than at room temperature. No definite reason for this condition can be ascertained, but a possible explanation can be made. At room temperature, the movement of the solvent was very rapid and might have been too fast for the components to partition between the two phases whereas, at the lower temperature the movement of the solvent was slow enough to allow for a proper distribution of the components between the immobile and mobile phases.

Equilibration at the lower temperature did not have any effect on the resolution of the tannin. It was observed that with or without equilibration the sorghum tannin resolved into two components when developed with methyl ethyl ketone:15% acetic acid (1:1) at the lower temperture.

The solvent systems in which other constituents were added to methyl ethyl ketone failed to effect any resolution of the tannin. These findings are illustrated for solvent systems 9 - 13 in Table XII.

Comparison of the Crude Extract and Partially Purified Sorghum Tannin

It was of interest to find out if there was any difference between the extract and purified tannin that could be detected chromatographically. A concentrated extract and a solution of isolated tannin from Sample #2 were compared using methyl ethyl ketone plus varying amounts of acetic acid. When methyl ethyl ketone saturated with 5% acetic acid was used at the lower temperature and with three hours of equilibration, the extract and purified tannin gave similar results. There were two spots, one having an R_f value of 0.4, the other at the solvent front. Only one spot was observed for both the extract and purified tannin when the chromatograms were

developed with the aqueous phase of methyl ethyl ketone saturated with 10% acetic acid at room temperature and without equilibration. Both the isolated tannin and extract produced two spots when the chromatogram was developed in the refrigerator without equilibration, using a methyl ethyl ketone:15% acetic acid (1:1) solvent system. These results indicated that both the extract and purified tannin were similar in their composition, and that they both contained the same materials which were positive to the arsenic-tungstate reagent. These results for three of the sorghum varieties are illustrated in Plate I.

Comparison of the Five Varieties

The isolated sorghum tannins from all five samples were chromatographed using the following solvent systems: methyl ethyl ketone:15% acetic acid (1:1); methyl ethyl ketone:10% acetic acid (1:1) and methyl ethyl ketone saturated with 5% acetic acid. They were all developed in the refrigerator without equilibration. With all three systems two components were resolved, the fast moving component having an R_f value of 1.0 and the slower moving component having different R_f values as shown in Table XIII. These R_f values were much lower with the saturated 5% acetic acid system and there was much more streaking than when the chromatograms were developed with the other two systems. When equal amounts of tannin from the five samples were combined using the 5% saturated system, two components were detected, one with an R_f value of approximately 0.3, the other at the solvent front. Other spray reagents, described earlier, were also used to ascertain whether or not there was any material present which was not detected by the arsenic-tungstate reagent. The results with the three sensitive reagents, i.e., ammoniacal silver nitrate, bis-diazotized benzidine and arsenic-tungstate,

were identical. From these results it was concluded that there was no difference in the tannin components among the five sorghum varieties that could be detected chromatographically.

TABLE XIII

R_f VALUES FOR THE SLOWER MOVING COMPONENT

Sample #	Solvent System 1	Solvent System 2	Solvent System 3
1	.75	.79	.40
2	.75	.74	.39
3	.75	.75	.37
4	.78	.77	.34
5	.79	.74	.38

Solvent System 1 - methyl ethyl ketone:15% acetic acid (1:1)
Solvent System 2 - methyl ethyl ketone:10% acetic acid (1:1)
Solvent System 3 - methyl ethyl ketone saturated with 5% acetic acid

Chromatography of the Individual Components

Since in earlier experiments the sorghum tannin was found to be resolved into only two components, an experiment was set up to find out whether or not either component was a degradation product of the other component. Ascending chromatography was employed, and the chromatograms were developed with methyl ethyl ketone saturated with 5% acetic acid under refrigeration and with no equilibration. Paper strips, 4" x 15", were employed in this experiment. A composite of the isolated tannin from all five samples was placed on the origin two inches from one end and the chromatogram developed. After the chromatogram was developed and dried,

one inch of this chromatogram was cut off, and the spots were identified by the arsenic-tungstate reagent. With the location of the spots on this strip as a guide, the spots on the remainder of the chromatogram were cut out, eluted with water under a closed system and chromatographed separately using the same solvent system and conditions.

Each component, when eluted and rechromatographed separately, had essentially the same R_f value that it had on the original chromatogram as shown in Plate I. From these results it was evident that neither component was a product of the degradation of the other during the course of the experiment. It was concluded from the results of this experiment that there were at least two definite "tannin components" in grain sorghum, and that neither component could be further resolved with this solvent system.

Legend to Plate I

PAPER CHROMATOGRAPHY OF GRAIN SORGHUM TANNIN

Solvent system - Methyl ethyl ketone saturated with 5% acetic acid.

Conditions - Refrigeration (+3°C) without equilibration.

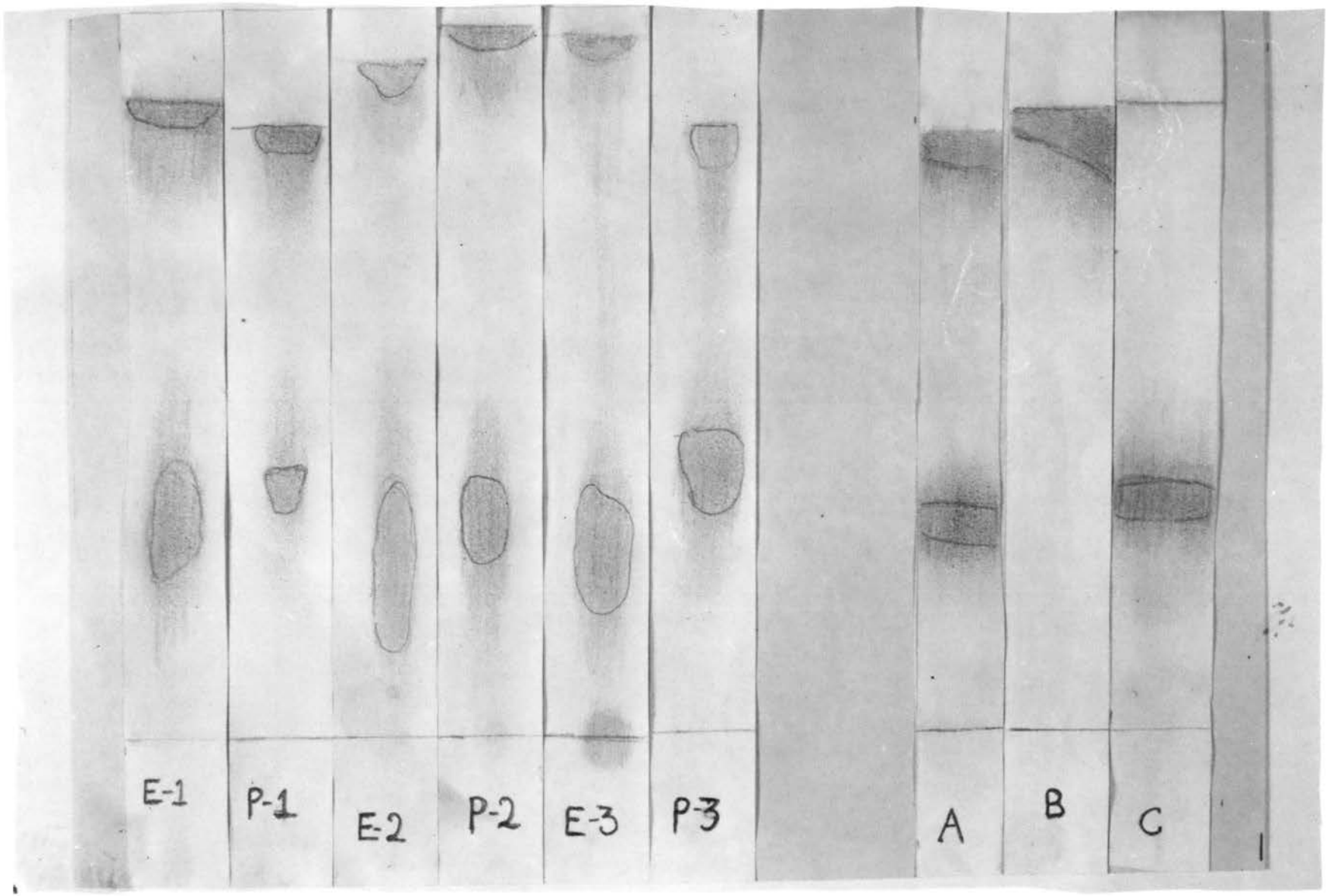
Comparison of Extract and Isolated Tannin

E-1 - Extract from Sample #1
P-1 - Isolated tannin from Sample #1
E-2 - Extract from Sample #2
P-2 - Isolated tannin from Sample #2
E-3 - Extract from Sample #3
P-3 - Isolated tannin from Sample #3

Chromatography of the Individual Tannin Components

A - Original chromatogram; R_f values of 1.0 and 0.35
B - Chromatogram of the fast moving component; R_f value of 1.0
C - Chromatogram of the slow moving component; R_f value of 0.37

Plate I



SUMMARY

Paper chromatographic techniques were used to study the composition of grain sorghum tannin. Both ascending and descending chromatograms were run using a wide variety of different solvent systems.

Of all the solvent systems investigated only one was found to resolve the tannin into two components. This was a methyl ethyl ketone:acetic acid: water system. Methyl ethyl ketone:plus 10 or 15% acetic acid (1:1) resulted in the separation of two reagent positive components. When the ketone was saturated with 5% acetic acid, the tannin resolved into two components, one having an R_f value of about 0.4, and the other one migrating with the solvent front. These results were obtained when the chromatograms were run at a low temperature (+3°C), either with or without equilibration, and could not be duplicated when the chromatograms were run at room temperature.

There was no appreciable difference between chromatograms of the crude extract and isolated tannin when they were chromatographed using the solvent systems described above. It was also observed that there were no differences among the isolated tannins from the five sorghum varieties. When the individual components were eluted and rechromatographed there were no degradation products from either component between the time the individual components were eluted and rechromatographed. This would indicate that neither component was a degradation product of the other component. It was observed that each component had moved as a single spot in a manner similar to its movement on the original chromatogram. It was concluded

from this experiment that in the isolated tannin there are at least two different components and that neither component could be further resolved using a methyl ethyl ketone saturated with 5% acetic acid solvent system.

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APPENDIX

A. Preparation of Color Reagents

1. Arsenic-tungstate Reagnet

a. 100 grams - sodium tungstate
b. 30 grams - arsenic acid
c. 300 ml. - distilled water
d. 50 ml. - concentrated hydrochloric acid

Reflux for two or three hours, cool and make up to 1 liter.

2. Folin-Denis Reagent

a. 100 grams - sodium tungstate
b. 20 grams - phosphomolybdic acid
c. 50 ml. - 85% phosphoric acid
d. 350 ml. - distilled water

Reflux for two hours, cool and make up to 1 liter.

B. Preparation of Spray Reagents

1. Ammoniacal Silver Nitrate Reagent

a. 20 ml. - 5% aqueous silver nitrate
b. 10% ammonium hydroxide

Add enough ammonium hydroxide to redissolve the silver oxide and dilute to 100 ml.

2. Bis-Diazotized Benzidine

a. 6 grams - benzidine hydrochloride
b. 14 ml. - concentrated hydrochloric acid
c. 980 ml. - distilled water
d. 650 ml. - 10% aqueous sodium nitrate

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