THE DETERMINATION OF STARCH IN GRASSES

By

ORVAL K. GLENN

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Northwestern State College

Alva, Oklahoma

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Thesis Approved:

James E. Webster
Thesis Adviser

George Odell
Faculty Representative

Dean of the Graduate School

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INTRODUCTION

Proximate feed analysis is a valuable tool in the evaluation of forages for ruminants, although it is not adequate for all purposes. Its usefulness is particularly limited in the crude fiber and nitrogen-free extract fractions, which are essentially carbohydrates. Several years ago an Experiment Station project was initiated to study the carbohydrate fractions of several western Oklahoma range grasses. The experiments discussed in this study represent one phase of the detailed carbohydrate analyses.

Many methods are available for the detailed study of these carbohydrate fractions. However, they are frequently not in agreement among themselves. This is due in some instances to lack of knowledge of the chemistry of components other than those being tested and in other cases accuracy is sacrificed in order to obtain a rapid determination. This is especially true for starch as the diastase method used in the project studies includes much more in its results than true starch. The values for starch found by the diastase method are useful in evaluating the nutritive value of forages, however it would be of scientific interest to determine the amount of true starch. The work covered in this report was instituted to obtain this information.

REVIEW OF LITERATURE

Although much is known about the chemistry of starch, very little is known about its distribution in the grasses.

The occurrence in grasses of non-starch polysaccharides, and the lack of knowledge of the chemistry of some of these fractions presents an obstacle which must be overcome in devising a method for the determination of true starch.

The monosaccharides and disaccharides occurring free in plant materials do not interfere with starch determinations. They are included in the soluble carbohydrate fraction and are extracted before starch is determined. This extraction can not be made with hot water as much of the starch would be included (22). The commonly used solvents are ethanol or cold water.

McIlroy, (11) in 1943, reported a new water soluble polysaccharide in the foliage of rye grass which he believed to be a glucose-galactose polymer. If ethanol were used as the extracting solvent for the removal of simple sugars, this polysaccharide would very likely be determined as starch.

One of the first methods devised for the quantitative estimation of starch was the acid hydrolysis method. In this method the sample was extracted with cold water or ethanol (5). The residue was then boiled for $2\frac{1}{2}$ hours with 200 mls. of water and 20 mls. of hydrochloric acid, cooled and nearly neutralized with sodium hydroxide, and the reducing

sugars determined as glucose. The amount of glucose multiplied by the factor 0.90 equals the amount of starch present in the original sample (5).

In grasses there are many other carbohydrates that undergo hydrolysis by this procedure, and these carbohydrates would be included in the fraction termed "starch by acid hydrolysis". Although this method does not give true starch percentages, it does give an estimation of the reserve carbohydrates (23).

With the failure of acid hydrolysis to give a value for true starch, experimenters turned to hydrolysis by enzymes because of their greater degree of specificity. Although enzymes from many sources have been studied, the most commonly used ones are malt diastase (15), Taka-diastase (3,5), and salivary amylase (16).

In most enzymatic procedures the plant material is extracted with cold water or ethanol. The residue is treated with the enzyme preparation which yields a mixture of glucose, maltose and perhaps dextrins; all of these are soluble in water and may be washed from the sample and the hydrolysis to glucose completed with acid. The glucose formed can then be determined by conventional methods for reducing sugars (16). It is difficult to obtain pure enzyme preparations and values obtained for starch by enzymatic hydrolysis upon plant material such as grasses as it will contain fractions other than pure starch. With samples of pure starch, both Taka-diastase and malt diastase work equally well and give results within the range of experimental error (3).

Several methods have been devised in which the starch, along with other plant carbohydrate components, are rendered soluble and selecti-

vely precipitated from solution.

Before extracting the starch, the plant material is extracted with either ethanol, cold water, ether, or a combination of one or more of these (19). The material must be ground very fine, preferably 100 mesh or finer (2). The starch extracting agents used may be cold dilute HCl (19), hot dilute KOH (2), boiling calcium chloride (4), alcoholic nitric acid (14), boiling dilute ammonium carbonate (21), or cold perchloric acid (18,10). Conditions should be kept mild enough to rupture the starch granules without hydrolyzing any of the starch. The most widely used and studied extracting agents are cold dilute HCl, hot dilute KOH, and cold perchloric acid (4.8N). The HCl will destroy some starch in one-half hour. Starch is believed to be fairly stable in hot, dilute KOH solution. Starch is stable in a calcium chloride solution (if the pH is kept within a narrow range) (2). Cold 4.8N perchloric acid does not destroy starch. At lower normalities it gives incomplete extraction; at higher normalities starch is lost through hydrolysis (10,13).

The starch must next be isolated from the extraction mixture. In 1927, Rask (19) proposed a method for determining starch based on the insolubility of starch in ethanol, which is added to the extraction mixture. The starch is precipitated out of solution, dried, weighed, and reported as starch. Rask claimed that cellulose, hemi-cellulose, and proteins will not contaminate this starch. Before extracting the starch, Rask extracted the sample with ether, ethanol, and cold water. This would eliminate many polysaccharides, including the one isolated by McIlroy (11). Any unknown polysaccharide in a solubility class with starch would be likely to contaminate this starch.

Chinoy (2) and others thought the most specific and accurate methods for starch should be based on its reaction with iodine. Chinoy claimed that starch-iodine is a definite addition product and that under standardized conditions, a definite quantity of iodine enters into combination with starch. Most starches are a mixture of amylose and amylopectin, with many starches containing from 22-26 percent amylose and 74-78 percent amylopectin. Exceptions are sugary mutant corn starch which may contain as much as 70 percent amylose, and waxy-maize starch which runs 95 percent or higher in amylopectin content. The amylopectins of waxy cereals are of lower molecular weight than amylopectins of most starches (24).

Amylose is the component of starch that is largely responsible for the blue color formed with iodine, adding about 20 percent of its own weight in iodine (24).

It has been demonstrated that all of the starch can be precipitated from solution with iodine, regardless of its amylose to amylopectin ratio (18). One precipitation with iodine has been found sufficient to precipitate all the starch in pure starch samples (18). Pectin, inulin, raffinose, protein, and glycogen are not precipitated by iodine (7,18), but contamination is possible.

Chinoy (2) suggests weighing the starch-iodine precipitate and multiplying by a factor found by the addition of iodine to a known amount of the same type starch under like conditions. The amylose to amylopectin ratio would be critical in this procedure.

Other workers remove the iodine through the use of alcoholic NaOH (7,17,18), releasing the free starch. The liberated starch may then

be weighed (4). The amylose to amylopectin ratio makes no difference in this procedure. Both of these methods are useful for the determination of starch in samples containing large amounts of starch. Also, after the starch has been liberated from the iodine it may be hydrolyzed by acid and the amount of glucose formed may then be determined as reducing sugars (18,6).

An anthrone-sulphuric acid reagent has been used to determine the amount of liberated starch (8). The acid hydrolyzes the starch to glucose, which reacts with the anthrone to form a green color. Poor reproducibility has been obtained using the anthrone reagent (9,10).

The liberated starch also may be dissolved in hot water, or other dispersing agent, and the optical rotation compared with standard amounts of starch (21). This method requires relatively large amounts of starch to be present in the samples.

After the liberated starch has been dissolved, it may again be treated with iodine and the blue color formed compared colorimetrically with standard solutions of starch treated in the same manner (6,17). The amylose to amylopectin ratio is critical in the procedure, making the proper choice of standard important.

Simerl and Browning (20) studied the spectra of starch-iodine using starch prepared from arrowroot, tapioca, canna, corn, potato, rice, rye, sargo, sweet potato, and wheat. The maximum absorption of these starches occurred from 560 to 640 millimicrons, with deviation of mean transmission curves being plus or minus 12 percent. They found that Beer's law was followed when a large excess of iodine was used.

Pucher, et.al.(17), found that the blue color formed was stable and reproducible when a large excess of iodine was added.

The solution in which the blue starch-iodine color is formed should be neutral or acidic (20).

METHODS

Preparation of samples.

The grass samples were secured from the Southern Great Plains Field Station at Woodward, Oklahoma where they were collected under the direction of Mr. E. H. McIlvain. Samples were collected from the range by hand in a manner to simulate grazing by cattle, i.e. they comprise that fraction of the grasses that cattle would graze at a given season of the year.

The samples were dried at 80°C. until constant weight was reached after which they were sent to Stillwater, Oklahoma for analysis. In the laboratory, the samples were ground to pass the medium screen of a Wiley cutting mill and then dried in an oven at 105°C.

Soluble sugars must be removed from the samples before they can be analyzed for starch and this was accomplished by extracting 10 to 15 gram samples in Soxhlets with 80 percent ethanol for 48 hours. The dried, extracted residues were ground in ball mills for 48 hours which reduced the samples to a fineness such that they would pass a 100 mesh screen.

Analytical methods.

Starch by acid hydrolysis (1). Two gram samples of the dry, powdered, extracted residue were weighed into 600 ml. Berzelius beakers, and 200 mls. of water and 20 mls. of HCl (sp. gr. 1.125) were added.

The samples were placed on a crude fiber digestion rack and boiled gently for 2½ hours. The samples were cooled and neutralized to methyl red with 50 percent NaOH. The samples were then filtered into 250 ml. volumetric flasks and washed sparingly to make to volume. Suitable aliquots were then used for the determination of reducing sugars by the Shaffer-Hartmann method (12).

Starch by diastase (1). One gram portions of the extracted samples were weighed out and placed in 150 ml. beakers, 50 mls. of hot water added, and the samples heated in a boiling water bath for 15 minutes with occasional stirring. The temperature of the water in the bath was lowered to 50-55°C. and 10 mls. of Taka-diastase solution containing 0.2 grams per 10 mls. were added to each of the samples. A blank also was started at this time. The samples were stirred and held at 50-55°C. for one hour. The samples were next made up to 250 mls. and filtered through Whatman number 54 filter paper. Two hundred ml. aliquots of the filtrates were measured into 600 ml. tall form beakers containing 20 mls. of HCl (sp. gr. 1.125). The beakers were placed on a hot plate and refluxed for 2½ hours. The samples and flasks were cooled in running water and the samples made just acid to methyl red by adjustment with concentrated HCl and NaOH. The samples were made up to 250 mls., filtered, and suitable aliquots taken for the determination of reducing sugars by the Shaffer-Hartmann method (12).

Starch by the starch-iodine method (Adapted from the method of Pucher, et. al. (18) with a colorimetric modification). Two hundred and fifty milligrams of each extracted sample were placed in 40 ml. centrifuge tubes. The samples were covered with washed sand, 4 mls. of water added, and the tubes were placed in a gently boiling water

bath for 15 minutes. The tubes were allowed to cool by placing in a water bath at 22-25°C. Three mls. of 72 percent perchloric acid were added with stirring, and glass rods were used to grind the samples against the tube walls, keeping the temperature between 22 and 25°C. This treatment was continued for 20 minutes, stirring constantly. Fifteen mls. of water were then added and the tubes were centrifuged until the plant material was settled firmly enough to allow the solutions to be decanted. The solutions were saved, the residues washed with water again by centrifuging, and the wash water added to the beakers. Four mls. of water were again added, the samples placed in the boiling water bath for 20 minutes, cooled, and treated with perchloric acid, as before. The liquids were added to those saved from the first extraction. Four mls. of water were again added to the residues and the tubes placed in a boiling water bath for 20 minutes. The tubes were again centrifuged, without the perchloric acid treatment, and this water added to the combined liquid extracts (at this point the residue may be tested for starch by adding a small amount of iodine in KI solution). If starch is found to be present, repeat the perchloric acid treatment. The solutions were diluted to 100 mls. with water. Twenty five mls. of these solutions were added to 40 mm. centrifuge tubes, along with 10 mls. of 20 percent NaCl solution, and 2 mls. of iodine in KI solution. The tubes were allowed to set until the blue color began to settle to the bottom and were then centrifuged until the blue starch-iodine was packed firmly in the bottom of the tubes. The supernatant liquid was carefully decanted and discarded. The starch-iodine precipitates were washed by being suspended in 10 mls. of

alcoholic NaCl, centrifuged, and the supernatant liquid decanted and discarded.

Two mls. of alcoholic NaOH were then added, and the tubes were gently tapped (do not try to stir with glass rods) until all blue color was discharged. Ten mls. of alcoholic NaCl were next added and the solutions centrifuged. The supernatant liquid was decanted and the purified starch washed with alcoholic NaCl.

Twenty five mls. of water were added to the starch, the tubes were placed in a boiling water bath, and the solutions stirred until all the starch was dissolved. These solutions were diluted to 50 mls. and two portions of from one to 25 mls. of each, depending upon the amount of starch present, were placed in colorimeter tubes and diluted to 25 mls. One ml. of iodine in KI solution was added to each tube and the color formed was read in an Evelyn photoelectric colorimeter, using a 565 millimicron filter, and compared to color formed in standard solutions prepared from a suitable starch. In this work soluble starch for indicator was used to prepare the standard solutions and the results obtained were divided by 1.11 to give the values quoted in Tables I and II (see discussion of starch-iodine method, this paper).

Reagents.

Iodine-potassium iodide solution. Seven and one-half grams of iodine plus $7\frac{1}{2}$ grams of potassium iodide were made up to 250 mls. with water and allowed to set over night. The solution was decanted into a glass stoppered bottle, filtering if necessary.

Alcoholic sodium chloride. Three hundred and fifty mls. of ethanol, 80 mls. of water, and 50 mls. of 20 percent aqueous NaCl were mixed,

and made up to 500 mls. with water.

Alcoholic sodium hydroxide (0.25N). Three hundred and fifty mls. of ethanol, 100 mls. of water, and 25 mls. of 5N NaOH were mixed and diluted to 500 mls.

Twenty percent sodium chloride solution. Fifty grams of NaCl were dissolved in water and made to 250 mls. volume.

Perchloric acid, 72 percent reagent grade.

DISCUSSION OF THE STARCH-IODINE METHOD

The colorimetric starch-iodine method gave very good checks on duplicate runs, and the precision seems to be excellent. The accuracy will depend upon the choice of standard. Soluble starch (for indicator) was used to prepare the standards.

Samples of commercially prepared arrowroot starch were dried at 100°C., weighed, and run through the complete method. The recovery was found to be 73 percent. This does not necessarily imply that starch was lost in the determinations, but it does indicate that soluble starch would not be the correct standard to use for the determination of arrowroot starch. The fact that very good checks were obtained on duplicate runs indicates that starch was not being haphazardly lost. If it were desirable to use arrowroot starch as a standard, dividing the results based on the soluble starch standards by 0.73 would give comparable results.

The first plan was to use arrowroot starch as a standard, since absolute accuracy would not be needed to follow the seasonal trends. It appeared that there would be very little starch in any of the samples. With the large amount of starch found in the September 21, 1953 sampling of sand dropseed, it became of interest to determine the exact amount of starch contained in this sample. This was accomplished by hydrolyzing with acid a portion of the solution containing the isolated starch, and then determining the starch from the glucose formed in the reaction.

The colorimetric method, based on the soluble starch indicator standards, gave 111 percent recovery of the starch found to be in this sample. By dividing the results based upon soluble starch standards by 1.11, the values obtained would be compared to sand dropseed grass starch. Both grasses studied were compared to this starch.

It is possible that the amylose to amylopectin ratio of grass starch may vary with the degree of maturity of the plant. Thus, complete accuracy is not assured even when using a standard prepared from starch of the particular grass. If the standard is prepared from a sample at the peak of the seasonal trend, however, the error would become less significant in the samples containing smaller amounts of starch.

The blue starch-iodine color would be expected to follow Beer's law only if the excess iodine were removed. When the excess iodine is not removed and the blank reading is made on water containing the amount of iodine added to the unknowns, the color formed would be expected to deviate from Beer's law since a part of the iodine is used to form the starch-iodine complex. This was observed when very small amounts of iodine were added. Beer's law was followed when larger amounts of iodine were added. One ml. of iodine in KI solution added to 25 mls. of solution gave a very large excess. A small fraction of this amount may be used, but the curve formed by plotting the logarithm of the percent transmission vs. concentration of starch will not be so steep, precision will be limited, and the color formed will not be reproducible when applied to solutions containing large amounts of starch. Adding a large excess of iodine intensifies the blue color. For this reason, care must be taken to keep the iodine content of the iodine in KI solution

as constant as possible while a series of determinations are being made. Store the solution in a brown, glass stoppered flask. If the flask is left unstoppered the iodine will escape from the solution. A new standard curve should be made if the iodine in KI solution is used over a long period of time, or when a new solution is prepared.

The addition of acid to the solution containing the starch-iodine dispersion will also deepen the color. However, it appears that it is not necessary to acidify the solution if all the base is carefully washed from the starch and a large excess of iodine used.

The colorimetric comparison of the isolated starch is fast and easily applied to samples that are very low in starch, and to samples that vary over a wide range of starch concentrations, as long as all the samples contain one type of starch.

The isolation of the starch from the plant material is very tedious for routine use and only a few samples can be run at one time. Care also must be taken to avoid loss of small particles of starch or starch-iodine during the numerous washings.

TABLE I

STARCH CONTENT OF INDIAN GRASS (SORGHASTRUM NUTANS) COLLECTED AT VARIOUS DATES AND STAGES OF MATURITY

(DRY WEIGHT PERCENTAGES)

Date	Description	A.H.* %	T.-D.* %	St.-I.* %
5/20/53	Green, 9" leaves.	31.14	4.43	0.42
7/24/53	Vigorous summery growth, 14" leaves.	27.06	2.53	0.19
9/21/53	Too dry for seed stalks, 11" leaves.	29.21	3.26	0.16
12/15/53	Dormant, 10" leaves.	30.94	5.71	0.00
1/14/54	Dormant, 7-8" leaves.	33.73	5.72	0.00
3/25/54	Dormant, 7-8" leaves.	31.64	5.84	0.00
5/18/54	Very green, 8" leaves.	25.73	3.89	0.51
7/16/54	Summer dormant 10% green, 12" leaves.	29.93	6.45	0.00
9/4/54	Dormant, 12" leaves.	33.31	7.85	0.00
11/17/54	Dormant, 12" leaves.	31.90	6.95	0.00
2/15/55	Dormant, 2" leaves.	28.70	5.02	0.00
3/17/55	Dormant, 2" leaves.	31.32	7.11	0.00
5/25/55	Green and tender, 14-16" leaves.	Sample was charred.		
7/20/55	No seed stalks, 21" leaves.	27.86	5.13	0.61

*A.H. (starch by acid hydrolysis).
T.-D. (starch by Taka-diastase).
St.-I. (starch by the starch-iodine method).

TABLE I (continued)

Date	Description	A.H. %	T.-D.%	St.-I.%
9/14/55	Heading, 25" leaves, 48" seed stalks.	29.90	5.27	0.61
11/22/55	Dormant, 20" leaves, 40" seed stalks.	28.07	3.99	0.18
2/16/56	Dormant 20" leaves, 40" seed stalks.	31.92	11.60	0.28
3/28/56	Dormant, trace of green, 20" leaves, 40" seed stalks.	28.46	3.56	0.23
5/23/56	Mid-spring growth, 13" leaves.	28.63	10.43	2.33

TABLE II

STARCH CONTENT OF SAND DROPSEED (SPOROBOLUS CRYPTANDRUS)

COLLECTED AT VARIOUS DATES AND STAGES OF MATURITY

(DRY WEIGHT PERCENTAGES)

Date	Description	A.H.* %	T.-D.* %	St.-I.* %
5/20/53	Green, 5-6" leaves.	27.59	6.18	1.86
7/24/53	Heading out, 21" seed stalk.	26.69	1.88	1.00
9/21/53	Ripe seed, 8" leaves, 22" seed stalks.	36.69	15.62	8.69
12/15/53	Dormant (to green), 20" leaves.	33.59	7.74	1.51
1/14/54	Dormant, 20" leaves.	31.44	6.54	0.96
3/25/54	Dormant, 20" leaves.	31.63	5.94	0.00
5/18/54	Very green, 3-5" leaves.	34.07	4.01	0.00
7/26/54	Seed ripe, plant 30% green, 5" leaves, 15" seed stalks.	28.01	6.33	0.39
9/4/54	Dormant and green, 5" leaves.	29.42	4.55	0.24
11/17/54	Dormant, trace of green, 8" leaves.	30.43	5.06	0.00
2/15/55	Dormant, 5" leaves.	26.71	6.24	0.00
3/17/55	Dormant, trace of green, 6" leaves.	32.79	6.25	0.00
5/25/55	Green and tender, 6-8" leaves. (charred)	22.67	5.67	0.00

*A.H. (starch by acid hydrolysis).
T.-D. (starch by Taka-diastase).
St.-I. (starch by the starch-iodine method).

TABLE II (continued)

Date	Description	A.H.%	T.-D.%	St.-I.%
7/20/55	Seed ripe, 4-14" leaves, 20" seed stalks.	31.18	8.34	2.12
9/14/55	Seed stalks dry, 7" leaves, 20" seed stalks.	29.72	7.97	2.29
11/22/55	Dormant, 3-5" leaves, 20" seed stalks.	32.65	10.87	2.75
2/16/56	Dormant, trace of green, 3-5" leaves, 20" seed stalks.	31.71	5.76	1.13
3/28/56	One inch of green, 20" seed stalks.	26.27	5.58	0.57
5/23/56	Drouth stunted, 6" leaves.	28.63	8.51	1.70

RESULTS AND DISCUSSION

The acid hydrolyzable, Taka-diastase hydrolyzable, and starch by starch-iodine fractions did not follow like trends at all times. However, when starch percentages found by the starch-iodine procedure showed large changes so did the values found by Taka-diastase and in many cases, those found by acid hydrolysis. As both the acid hydrolyzable and diastase hydrolyzable fractions appear to contain components other than starch, there would not necessarily be a correlation between them.

The starch by starch-iodine (referred to in the following as starch) followed a similar trend in both the grasses studied although the pattern varied between the two. Indian grass contained starch while it was growing vigorously, but when growth became dormant the starch dropped to zero in most instances. Sand dropseed developed starch later in its growing period and retained starch later into the fall.

Indian grass contained less starch than sand dropseed. During the 1953 growing season it lost starch slowly, reaching zero amounts by January. The sampling of May 18, 1954 was the only one of that year found to contain starch. By the time of the next sampling in July, the grass was described as summer dormant and the starch content was back to zero with no starch being found until the growing season of 1955. Although only slightly more starch was formed in 1955 than in the two preceeding years, the starch did not vanish at the end of the growing

season and made a rapid climb during the 1956 growing season. The 1955 growing season was the first during this study favorable enough to allow Indian grass to form seed. During 1953 and 1954 the grass had never grown over 14 inch leaves and had not formed seed stalks. In 1955 it grew 20 inch leaves and 40 inch seed stalks. The behavior of the grass during these two seasons indicated that much different results would have been obtained had the study been made over a period of more favorable conditions.

Sand dropseed apparently found the growth conditions more favorable than did Indian grass. It formed seed each year of this study. The May 20, 1953 samples contained starch, but the percentage dropped at the July sampling. In September the seed was ripe and the plant contained the greatest amount of starch of the 1953 growing season. The starch content then dropped off, reaching zero amounts by March, 1954. The next sample found to contain starch was taken in July, 1954, at which time the seed was ripe and the plant again contained the largest amount of starch for the season, although only a fraction of the amount found the year before. In 1954 the grass only grew 15 inch seed stalks, compared with 22 inch stalks in 1953. The starch content dropped rapidly, reaching zero by November (note starch content of Indian grass grown in 1954). Starch was not found again until July 1955. The seed was ripe in July. However, the amount of starch continued to rise until November, declining thereafter, but never reaching zero this season (again note behavior of Indian grass).

In both grasses studied a comparison of the seasonal trends of the

results found by acid hydrolysis, Taka-diastase, and starch by starch-iodine show interesting similarities. Starch by starch-iodine appeared to follow most closely changes in vegetative growth of the grasses.

SUMMARY AND CONCLUSIONS

A study was made of selected methods for the determination of starch.

Comparisons were made of the acid hydrolysis, Taka-diastase, and starch-iodine methods for starch as applied to two western Oklahoma range grasses. Starch determined by acid hydrolysis varied from 22 to 36 percent. Starch determined by Taka-diastase varied from 1.8 to 15 percent, and starch by starch-iodine varied from 0.0 to 9 percent. Starch determined by the starch-iodine method followed definite seasonal trends and the amount was greatly influenced by conditions encountered in separate seasons.

The large percentages of acid hydrolyzable material, as compared to true starch, indicates very clearly that much work remains to be done to determine what are the individual components of this fraction.

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VITA

Orval K. Glenn

Candidate for the Degree of

Master of Science

Thesis: THE DETERMINATION OF STARCH IN GRASSES

Major Field: Chemistry

Biographical:

Personal data: Born near Cherokee, Oklahoma, January 24, 1932, the son of Roy O. and Thelma Glenn.

Education: Attended grade schools at Burlington, Oklahoma and Aline, Oklahoma. Attended high schools at Aline and Cleo Springs, Oklahoma, graduating from Cleo Springs High School in 1950. Received the Bachelor of Science degree from Northwestern State College, Alva, Oklahoma in May, 1954 with a major in chemistry. Graduate student at Oklahoma Agricultural and Mechanical College 1954-56.

Date of Final Examination: September, 1956.

THESIS TITLE: THE DETERMINATION OF STARCH IN GRASSES

AUTHOR: Orval K. Glenn

THESIS ADVISER: Dr. James E. Webster

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TYPIST: Virginia M. Vela