# A STUDY OF THE CARBOHYDRATES OF BIRD'S-FOOT TREFOIL (LOTUS CORNICULATUS)

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ABSTRACT

A STUDY OF THE CARBOHYDRATES OF BIRD'S-FOOT TREFOIL (LOTUS CORNICULATUS)

by Min-Gee Hsia

Four different solvents, namely, 80 percent ethanol, water, 0.2 N, and 0.8 N sulfuric acid were utilized to extract free and combined carbohydrates from bird's-foot trefoil (Lotus corniculatus). The extracts were clarified, dionized and silylated in preparation for gas-liquid chromatographic analyses.

Free sugars were identified as erythrose, mannose, fructose, glucose, galactose and a trace of i-inositol.

The polysaccharides in this legume are similar to those found in clover, but different than those that occur in grass. The polysaccharides present in bird's-foot trefoil are pentosan (water insoluble araban or xylan), hemicellulose (arabinoxylan or arabinogalactan) and hexosan (mannan, galactomannan or glucose polysaccharide other than starch). Neither starch, fructosan nor sucrose were detected in this forage. Based on the weight of dry tissue the first and second harvest contained 10.6 and 16.7 percent of total available carbohydrates and 6.5 and 9 percent of free sugars, respectively.

Approved: Harold M. Bell

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# A STUDY OF THE CARBOHYDRATES OF BIRD'S-FOOT TREFOIL (*LOTUS CORNICULATUS*)

By

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# INTRODUCTION

Carbohydrates are the primary source of reserve energy stored in the vegetative organs of biennial and perennial forage plants.

The study of the free and combined sugars of bird's-foot trefoil (Lotus corniculatus) was an attempt to further understand the chemical composition and the nutritive value of this perennial legume which was found recently to be more nutritious than many of the other legumes. Also, the extraction and characterization of the free and combined sugar of L. Corniculatus were essential steps in studying its metabolism in rumen digestion. Since the sugar contents of this legume is very low, the application of improved gas-liquid chromatographical techniques has achieved the successive identification of several free sugars from L. Corniculatus.

## LITERATURE REVIEW

The commonly used term "Bird's-foot Trefoil," referring to those species of the genus Lotus that are of agricultural importance, includes: Lotus corniculatus L, together with its numerous varieties; viz., L. uliginosus Schk (L. major Sm.), L. angustissimus L; and L. hispidus Desf.

Lotus corniculatus, var. tenuifolius, was first identified by Brand (4) in 1898. He indicated that L. corniculatus was the first lotus to be described by Bock (4) in the middle of the present century, this legume does not occur naturally in America, but is native to Europe, Australia and some parts of Asia and Africa.

Bird's-foot trefoil, a long-lived perennial of possible agricultural value, is more persistant than is red clover or alfalfa and is more drought-resistant than is any shallow-rooted legume such as white clover. Chemical analyses of the crops indicate that insofar as nitrogen, calcium, phosphorus and lignin were concerned, bird's-foot trefoil is equivalent in nutritive value to alfalfa for hay and white clover for pasture. Hence, it presents distinct possibilities for hay and pasture production on secondary or poorer soils where alfalfa is not successful, where red clover is too short-lived for rotation, and where

summer conditions are too droughty for the successful growth of white clover.

Carbohydrates in the form of sugars, starch, cellulose, hemicelluloses, pentosans, lignin, pectin and other carbohydrate-like material are present in larger amounts in forage crops than any of the other constituents.

M. Henrici (3) reported that starch was present in all fodder plants, as was sucrose, which was present in larger amounts than were the reducing sugars. Wilting and curing processes resulted in large decreases in starch content. The analytical studies on the carbohydrates of grass and clover by Laidlaw and Reid (14) revealed that glucose, fructose, sucrose and oligosaccharides occurred in grass and clover. Galactan, araban and glucose polysaccharides rather than the fructosans occurred in clover. The fructosan content of the grass can be readily obtained when the analysis is performed immediately after freeze-drying and milling of the sample. Enzymatic hydrolysis of sucrose and the fructosans occurred after being held under moist conditions. These carbohydrates also break down during wilting and ensiling, partially enzymic and partially acidic as a result of ensiling.

Harwood (2) isolated D-mannitol from perennial rye grass (Lolium perenne).

On the basis of feed analyses of grass and legume, Plummer (5) divided the carbohydrate constituents into

lignin which is indigestible, cellulose intermediate in digestibility and "other carbohydrates" representing the most easily digested fraction. He reported that the percentage composition of bird's-foot trefoil was: protein 20, fat 3.59, fiber 23.04, ash 6.05, nitrogen free-extract 47.32, lignin 7.73, cellulose 25.50, and other carbohydrate 37.13. Of the total percentage of carbohydrates, only an insignificant amount was free sugar.

Wilkins, Lindahy, Davis and Reynolds (13) determined the free-reducing, acid hydrolyzable and total sugars, and total available carbohydrate in Ladino clover. They reported that the carbohydrate contents of Ladino clover was affected by such factors as plant part, season, and time of day. By studying the carbohydrate reserves in alfalfa, red clover and bird's-foot trefoil in 1962, Smith (8) reported that alfalfa maintained the highest level of carbohydrate reserve which was followed by red clover. Very low carbohydrate reserves were maintained in bird's-foot trefoil. After a marked decrease during early spring, the carbohydrate reserve in bird's-foot trefoil remained at a low level under each management system until restored during the fall months.

Smith, Paulsen and Raguse (9) extracted total available carbohydrates from grass and legume tissues. They reported that alfalfa stored most of its reserve carbohydrates as starch in the roots, timothy stores the largest

proportion of its reserve carbohydrates as fructosans in the stem base.

Strepkov (10) made a complete scheme of analyses and divided the carbohydrate complex from plants into 6 groups:

(1) Carbohydrates soluble in hot ethanol (invert sugar, fructose, maltose, fructosides, galactosides and trehalose).

(2) Carbohydrates soluble in cold water but insoluble in alcohol (pectin substances, dextrin, gums and B-amylos).

(3) Carbohydrates soluble in water at 45-47°C (inulin).

(4) Carbohydrates which are hydrolyzed by diastase (starch).

(5) Carbohydrates soluble in hot water (xylan, araban and protopectin).

(6) Carbohydrates which undergo cleavage by treatment with 2 percent sulfuric acid (mannan, galactan, pentosan and amylan).

The application of gas-liquid chromatographic techniques to the separation of sugar and related polyhydroxy compounds has been used recently. A simple and rapid quantitative method for the preparation of O-trimethylsilyl (TMS) derivatives of sugar is now available and potentially useful for gas-liquid chromatography (11). The preparation of TMS derivatives is rapid and convenient and can be used on a micro scale. The analysis of plant materials for car-

bohydrates by gas-liquid chromatography (GLC) has become a practical laboratory procedure.

# EXPERIMENTAL

## 1. Preparation of Samples

Two samples of bird's-foot trefoil (L. Corniculatus) were collected from the first harvest on July 4, 1962 and one from the second harvest on July 16, 1962 at the experimental farm at Michigan State University. After the first cutting the hay became wet on the field from rain. Both samples were air dried in the field and baled after which it was further dried in a barn dryer with heated air (38-65°C).

In the laboratory the dried bird's-foot trefoil material was ground to pass through an 80-mesh sieve, placed in a glass container and oven dried over phosphorus pentoxide at 105°C to constant weight. The bottles were tightly capped and stored for analysis.

## 2. Extraction of Total Available Carbohydrates

The extraction of both free and combined sugars was made by two methods: (1) water extraction, and (2) acid extraction.

### Water Extraction

Samples of bird's-foot trefoil (4 g) were extracted with 150 ml of water in a Soxhlet apparatus for 3 hours.

The hot aqueous solution was poured first through a Schleicher and Schnell (S & S) filter paper and then through a Whatman No. 42 filter paper. The residues were washed with 50 ml of distilled water and the extracts were combined and diluted to volume with water.

Part of the water extracts were then hydrolyzed by adding 2 ml of 25 percent solution of hydrochloric acid and then neutralized with sodium hydroxide.

Both the hydrolyzed and non-hydrolyzed water extracts were then subjected to clarification and deionization.

Acid Extraction

Extractions were made with both 0.2 N and 0.8 N sulfuric acid. Each sample was placed in a 200 ml round bottom flask containing 50 ml of acid and refluxed for 60 minutes in a Glas-col heating mantle. A reflux condenser together with a Soxhlet condenser was used to prevent evaporation of the solvent.

The hot solution was filtered through a Whatman No. 42 paper, cooled, neutralized with sodium hydroxide solution and diluted to volume with distilled water.

3. Extraction of Free-Sugar by Alcohol Extraction

Free sugars were removed from the samples by extracting them with 50 percent, 80 percent and 90 percent aqueous ethanol. Extraction with hot 80 percent ethanol in a Soxhlet extraction apparatus for a short period proved to

be most efficient in removing all of the monosaccharides, especially the six-carbon sugars.

Accordingly 4-gram samples were extracted with 80 percent ethanol in a Soxhlet extraction apparatus for 3 hours, and the hot extracts filtered through S & S filter paper. Treatment of the alcoholic extract with most reagents for clarification gave only partial clarification. Therefore, before removal of the impurities from the extracts an equal volume of water was added and the solution concentrated under reduced pressure to remove most of the ethanol.

4. Clarification

In preliminary experiments several clarification procedures were evaluated.

Lead Acetate Method

Two milliliters of lead acetate solution were added to the extract, mixed and allowed to stand for 1 hour. It was filtered through a S & S filter paper into a beaker containing 2 g of potassium oxalate. The residue was washed 3 or 4 times with water, stirred occasionally, and permitted to stand for 1 hour.

Chloroform-Butanol Method

The aqueous extracts were shaken with chloroform and butanol to remove the protein (7).

Cadmium Hydroxide Method

The extracts were heated to 95°C and 10 ml of a 10 percent cadmium sulphate solution and 5 ml of a 0.5 N sodium hydroxide were added simultaneously with efficient stirring. The cadmium hydroxide was precipitated from the solution. The mixture was kept at 95°C for three minutes and was then cooled rapidly and filtered through Filter-Cel filter by use of a water aspirator.

This method gave better clarification since the gas-liquid chromatogram showed that most of the interfering substances had been removed. Hence, this method was adopted for the clarification of all extracts.

5. Deionization

The effective methods for deionization employed the use of Amberlite resin columns. In this method, IR-120 cation exchange resin and IR-4B anion exchange resin were used. The strongly acid condition which developed in the solution after passing through the IR-120 column may cause some hydrolysis and change the nature of the carbohydrates. This was minimized by using several sets of short columns in a series. Four columns were packed (5 cm x 1 cm for each column) with resins, two of them were filled with IR-120 and the other two with IR-4B. Samples of the finely-ground tissues were extracted and clarified as previously described, and the solutions were allowed to drip slowly

through the columns of IR-120 and IR-4B alternatively. This procedure was found to be most satisfactory for minimizing decomposition and transformation of the sugars.

## 6. Gas-Liquid Chromatography of Sugars

### Materials

Pyridine (Reagent Grade) was redistilled over barium oxide and stored over potassium hydroxide pellets.

Hexamethyldisilazane was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wisc.

Trimethylchlorosilane was obtained from General Electric Co., Silicone Product Division, Waterford, N. Y.

The column packing for gas-liquid chromatography was prepared from SE-30 (3 percent) or 15 percent polyethylene glycolsuccinate (EGS) on 80-100 mesh chromosorb W purchased from the Applied Science Corporation, State College, Pennsylvania.

The gas-liquid chromatography apparatus was a F & M Biomedical Model 400, equipped with hydrogen flame detector. Helium was used as the carrier gas. The column was 6 ft long and had an inside diameter of 4 mm. The flow rate of the carrier gas was held constant at 40 ml per minute.

The Amberlite IR-120 resin from Fisher Scientific Company (Nuclear Sulfonic, Acid type) and the Amberlite IR-4B from the Resinous Products & Chemical Company,

Philadelphia 5, Pa. were used for deionization.

Method for Trimethylsilylation

The trimethylsilylation method for the sylation of the sugars was identical to the one described by Wells, Pittman and Wells (12). The trimethylsilylation reagent (TMSi) was prepared by mixing pyridine, hexamethyldisilizane and trimethylchlorosilane in the proportions of 17:2:1 (V/V/V).

The standard solutions of glucose, mannose, fructose, galactose and other mono- or disaccharides were prepared separately by dissolving 100 mg of each sugar in 100 ml of water saturated with benzoic acid. One milliliter of the stock solution was evaporated at 50°C to dryness in a vacuum evaporator. One milliliter of TMSi reagent was reacted with the dry sugar residue in a 5 ml round bottom flask at room temperature. The mixture was shaken vigorously for about 1 minute, and allowed to stand for 5 minutes or longer at room temperature prior to chromatography. The remainder of the solution was stored in a small plastic stoppered glass vial for further use. (Solutions thus treated were found to be stable for several days to several months, depending on the kind of sugar, the dryness of the sugar residue upon trimethylsilylation, and the effectiveness of the TMSi reagent.)

One-ml portions of the clarified and deionized extracts were evaporated to dryness in a flash evaporator.

One milliliter of TMSi reagent was added to the dry residue and the reaction was made as previously described. The mixture was shaken vigorously for 15 minutes and allowed to stand for 1 hour before chromatography. Direct injection into the gas-liquid chromatography apparatus was made with a 10-µl Hamilton syringe, range from 0.1 µl to 0.5 µl.

The TMSi solutions were cloudy because of the precipitation of some ammonium chloride on the addition of trimethylchlorosilane. The TMSi-sugar derivatives were especially cloudy. These precipitates were found to cause no interference with the gas-liquid chromatogram, thus no further attempt was made to remove them.

Some carbohydrates dissolved with difficulty in the TMSi reagent. In these cases the dry carbohydrate residues were first treated with pyridine by warming the mixture to 90°C until most of the carbohydrate had dissolved. This was followed by the addition of hexamethydisilizane and subsequently with trimethylchlorosilane in a ratio 17:2:1 (V/V/V).

Preparation of the Alcohol Derivative

The preparation of the alcohol derivative from the corresponding aldose sugar was done by reduction of the sugar with potassium borohydride ($KBH_4$). One milliliter of the extract from the bird's-foot trefoil was cooled in an ice bath and then treated with 5.5 g of potassium borohydride. The reaction was carried out in a 10 ml test

tube and the mixture was allowed to warm to room temperature and to stand for 40 minutes before the addition of 0.1 ml of 1 N hydrochloric acid.

When the decomposition of the excess $KBH_4$ was complete, 0.6 ml of distilled water and about 400 mg of Dowex 50 X were added in that order. The supernatant was removed with a pipet and the remaining resin was washed with a 0.5 ml portion of water. The supernatant together with the water washings were combined and evaporated to dryness in a flash evaporator. The dry residue was treated with 1 ml of methanol and again evaporated in vacuo. The residue was washed 4 or 5 times with methanol. The final dry residue was reacted with TMSi reagent for gas-liquid chromatography.

## 7. Thin-Layer Chromatography of Sugars

In the preliminary experiment, several different thin-layer systems were studied for the separation and identification of the sugars in the sample extracts. None of these methods proved useful in the separation of the individual sugars. Hence, use of the procedure described by Kurt Randerath (6) was adopted since it proved most effective for clarifying the extracts. The plates were layered with Kieselguhr G buffered with acetate (E. Stahl and U. Kaltenbach).

Thirty grams of Kieselguhr G was mixed with 60 ml

of aqueous 0.02 M sodium acetate buffer and spread on the plates to a thickness of 0.25 mm and then dried at 100°C for 30 minutes. The thin-layer was developed by a solvent system containing 65 ml of ethyl acetate and 35 ml of a mixture of 2 volumes of reagent grade isopropanol and one volume of distilled water.

After development, the plates were sprayed with an indicator consisting of 95 percent ethanol, sulfuric acid and anisaldehyde in a ratio of 18:1:1, and then dried at 90-100°C for 5-10 minutes.

## RESULTS AND DISCUSSION

Four different methods were studied for the extraction of free and combined carbohydrates from bird's-foot trefoil tissues. However, the type of extraction used greatly influenced the quantitative values since the various carbohydrates stored in the plant have different configurations and different solubilities.

Extraction of samples of the second harvest with 0.8 N sulfuric acid gave 167.74 mg of total carbohydrates per gram of dry tissue; with 0.2 N sulfuric acid 109.34 mg; with water 89.96 mg; and with 80-percent ethanol 92.5 mg. Each value was the average of four extractions.

Extraction with 0.8 N sulfuric acid gave values which were considerably higher than those obtained with the other methods. Stronger acid solutions probably extracted structural carbohydrates, such as hemicellulose, polysaccharides, as well as free-sugars, fructosan, pentosan, and some oligosaccharides. Thus, 0.8 N sulfuric acid gives complete hydrolysis of most oligosaccharides and hydrolyzed hemicellulose and polysaccharides in a large degree, but some monosaccharides such as erythrose and fructose may undergo decomposition with this strong acidic treatment. Gas-liquid chromatograms of their trimethylsilyl ethers

showed that the 0.8 N sulfuric acid extract contained erythrose, arabinose, xylose, fructose, mannose, glucose, galactose and a small amount of i-inositol.

The amount of each individual sugar in mg per gram of dry tissue from the four types of extraction methods are listed in Tables I and II, and their ratios and percentages are given in Table III. Extraction with 0.2 N sulfuric acid gave smaller values for these constituents than those obtained with 0.8 N sulfuric acid. They superimposed each other on the peak location (retention time), but not on the peak area.

0.2 N sulfuric acid extracted all the free-sugars, fructosan, some pentosan, oligosaccharides, and polysaccharides; it cannot completely hydrolyze some oligosaccharides, and polysaccharides. For instance, the glucose-galactose linkage (raffinose type) is more resistant to acid hydrolysis and therefore the 0.2 N sulfuric acid may not hydrolyze this type of linkage completely as did 0.8 N sulfuric acid. As one compares the quantity of glucose and galactose in both acid extracts, 0.2 N sulfuric acid extraction gave values considerable less than those obtained with 0.8 N sulfuric acid. The values for arabinose and xylose showed the same tendency and indicated the presence of some pentosans (araban or xylan) or some hemicelluloses (arabinoxylan or arabinogalatan) which had not been completely hydrolyzed by 0.2 N sulfuric acid.

The amounts of mannose were also less than those from strong acid extract. This was probably due to some galactomannan or mannan which did not undergo complete hydrolysis by weak acid. On the other hand, the 0.2 N sulfuric acid extract gave erythrose values much higher than those obtained with stronger acid. In comparing the values for sugars in the two acid extracts from both harvests, one observes that 43 percent of the erythrose was decomposed by the stronger acid.

Water extractions with hot water removed all free sugars, fructosans, and some water soluble oligosaccharides, hemicelluloses and pentosans.

The gas-liquid chromatograms of the trimethysilyl ethers of the sugars in the aqueous extracts indicated the presence of erythrose, fructose, mannose, glucose and trace amounts of xylose and galactose. When the sugar contents of the water extracts and the acid extracts are compared, the lack of arabinose and xylose indicated the absence of some water-soluble pentosans. The total carbohydrates in the water extracts were about 20 percent less than those found in the sulfuric acid extracts. This difference may be due to some water insoluble araban or xylan and probably some arabinoxylan which was soluble in acid but was not in water. Erythrose and mannose were present in almost equal amounts in both water and 0.2 N sulfuric acid extracts.

Six water extracts were made, four of which were hydrolyzed with acid and subsequently neutralized and the

other two remained unhydrolyzed which were used as controls. No apparent difference was noted between the gas-liquid chromatogram of the hydrolyzed and the non-hydrolyzed extracts. The failure to increase fructose content in the hydrolyzed extracts indicates that no fructosan was extracted. Similarly, the lack of an apparent increase of glucose content in 0.2 N sulfuric acid extract as compared to the hydrolyzed water extract indicated that no starch was extracted. Bird's-foot trefoil, therefore contains entirely different polysaccharides than do the grasses, but are similar to the carbohydrates in clover, since no fructosan and starch were detected in clover.

The 80 percent ethanol extracts yielded erythrose, fructose, mannose, glucose, galactose and i-inositol. These were all of the free-sugars present in bird's-foot trefoil, with a preponderance of mannose. The quantities of mannose and fructose were much greater than those of other sugars found in the extracts. This may be explained on the basis that the alcohol extract was not subjected to acid hydrolysis and neutralization. The latter treatment could have decomposed some of the mannose and fructose.

Some difficulty was encountered in the separation of the α-mannose and the α-fructose peaks on the gas-liquid chromatograms. Since both sugars have very close retention times on both polar and non-polar columns, the large content of each sugar gives broader peaks and consequently the

two peaks converge at almost the same location. However, by using SE-30 columns and varying the temperatures, they could be separated. The interchangeable relationship of these two sugars occurs as indicated by the change of the ratio of both peaks. The quantitative values obtained for the average of four sets of data may have some slight errors.

The branched-chain five-carbon sugar apiose, which exists in some perennial rye grasses and clovers, was also expected to be present in bird's-foot trefoil. The analysis for this sugar was made by two methods:

I. Thin-Layer Chromatography - The sample was spotted on a silica gel plate, developed in pyridine: ethyl acetate: acetic acid: water (5:5:1:3 V/V) and developed with aniline hydrogen phthalate spray reagent.

II. Gas-Liquid Chromatography - TMS-derivatives of the sample residues were injected in the polyethylene glycosuccinate (PEGS) columns.

The results of both methods were negative and indicated the absence of apiose in the above-ground portions of bird's-foot trefoil plants.

A thin-layer chromatogram method described by Kurt Randerath (6) was tried on alcoholic extracts of bird's-foot trefoil tissue to determine the presence of free-sugars. Erythrose, mannose, galactose, glucose, and fructose were thus separated and identified.

The 80 percent ethanolic extract was reduced by potassium borohydride ($KBH_4$). All free-sugars were reduced to the corresponding alcohols in large proportions as noted by the comparison of their GLC chromatograms before and after reduction. Apparently mannose, fructose and glucose peaks decreased after reduction, because two new peaks were located and identified as mannitol and sorbitol.

In comparing the two harvests of bird's-foot trefoil, it was observed that the second harvest contained almost 30 percent more of both the free-sugars and total carbohydrates than did the first harvest.

TABLE I

AMOUNTS OF INDIVIDUAL SUGARS IN PLANTS FROM THE FIRST HARVEST OF L. CORNICULATUS AS DETERMINED BY GAS-LIQUID CHROMATOGRAPHY

| | Mgs. per gram of dried tissue | | | |
|---|---|---|---|---|
| Extraction Solvent | 0.8 N $H_2SO_4$ | 0.2 N $H_2SO_4$ | $H_2O$ | EtOH |
| α-Erythrose | 7.63 | 9.72 | 11.92 | 8.13 |
| β-Erythrose | 9.36 | 19.82 | 15.02 | 3.16 |
| α-Arabinose | 5.67 | 2.58 | ----- | ----- |
| β-Arabinose | 7.72 | 2.64 | ----- | ----- |
| s-Arabinose | 0.63 | | | |
| α-Xylose | 12.64 | 0.89 | 0.71 | ----- |
| β-Xylose | | 0.70 | trace | ----- |
| α-Fructose | 13.69 | 7.00 | 4.81 | 6.45 |
| β-Fructose | trace | 0.81 | 2.04 | 0.43 |
| α-Mannose | 20.59 | 19.13 | 20.09 | 30.55 |
| β-Mannose | 4.86 | | | 1.91 |
| α-Glucose | 8.42 | 5.58 | 3.34 | 5.66 |
| β-Glucose | 9.68 | 4.43 | 2.75 | 7.41 |
| Galactose | 4.84 | 1.58 | trace | 1.35 |
| i-inositol | 1.09 | 0.86 | 0.37 | 0.28 |
| Total | 106.82 | 75.74 | 61.05 | 65.33 |

TABLE II

AMOUNTS OF INDIVIDUAL SUGARS IN PLANTS FROM THE SECOND HARVEST OF L. CORNICULATUS AS DETERMINED BY GAS-LIQUID CHROMATOGRAPHY

| | Mgs. per gram of dried tissue | | | |
|---|---|---|---|---|
| Extraction Solvent | 0.8 N $H_2SO_4$ | 0.2 N $H_2SO_4$ | $H_2O$ | EtOH |
| α-Erythrose | 13.60 | 18.50 | 17.61 | 12.25 |
| β-Erythrose | 10.52 | 22.18 | 22.20 | 4.56 |
| α-Arabinose | 9.30 | 3.84 | ----- | ----- |
| β-Arabinose | 14.62 | 5.32 | ----- | ----- |
| s-Arabinose | | | | |
| α-Xylose | 16.86 | 4.65 | 1.07 | ----- |
| β-Xylose | trace | trace | ----- | ----- |
| α-Fructose | 12.55 | 7.64 | 3.65 | 10.17 |
| β-Fructose | trace | trace | 4.11 | 0.81 |
| α-Mannose | 33.95 | 29.17 | 29.61 | 46.31 |
| β-Mannose | 8.01 | | | 1.37 |
| α-Glucose | 17.15 | 8.05 | 5.25 | 8.92 |
| β-Glucose | 18.82 | 6.08 | 4.18 | 4.55 |
| Galactose | 9.52 | 2.64 | 1.23 | 1.61 |
| i-inositol | 2.84 | 1.27 | 0.55 | 1.98 |
| Total | 167.74 | 109.34 | 89.46 | 92.53 |
| First Harvest / Second Harvest | 63.67% | 68.36% | 68.23% | 70.60% |

TABLE III

BOTH FREE AND COMBINED MONOSACCHARIDE SUGARS IN PLANTS FROM THE FIRST AND THE SECOND HARVEST OF L. CORNICULATUS AS DETERMINED BY GAS-LIQUID CHROMATOGRAPHY

| Fraction | 0.8 N $H_2SO_4$ | | 0.2 N $H_2SO_4$ | | $H_2O$ | | EtOH | |
|---|---|---|---|---|---|---|---|---|
| Harvest | I | II | I | II | I | II | I | II |
| | % | | % | | % | | % | |
| α-Erythrose | 7.14 | 8.10 | 13.00 | 16.90 | 19.50 | 19.70 | 12.44 | 13.24 |
| β-Erythrose | 8.76 | 6.27 | 26.50 | 20.29 | 24.60 | 24.80 | 4.84 | 4.93 |
| α-Arabinose | 5.30 | 5.54 | 3.44 | 3.50 | - | - | - | - |
| β-Arabinose | 7.23 | 8.71 | 3.54 | 4.86 | - | - | - | - |
| s-Arabinose | 0.58 | | | | | | | |
| α-Xylose | 11.84 | - | 1.20 | - | 1.16 | 1.19 | - | - |
| β-Xylose | - | 10.05 | 0.94 | - | trace | trace | - | - |
| α-Fructose | 12.82 | 7.48 | 10.22 | 6.99 | 7.88 | 4.08 | 9.87 | 10.99 |
| β-Fructose | trace | trace | 1.08 | | 3.35 | 4.60 | 0.67 | 0.87 |
| α-Mannose | 19.27 | 20.23 | 25.59 | 26.68 | 32.90 | 33.10 | 46.77 | 50.04 |
| β-Mannose | 4.55 | 4.78 | trace | trace | trace | trace | 2.92 | 1.47 |
| α-Glucose | 7.88 | 10.22 | 7.47 | 7.37 | 5.46 | 5.87 | 8.67 | 9.64 |
| β-Glucose | 9.06 | 11.22 | 4.58 | 5.56 | 4.50 | 4.67 | 11.33 | 4.92 |
| Galactose | 4.53 | 5.67 | 2.12 | 2.41 | trace | 1.38 | 2.06 | 1.74 |
| i-inositol | 1.08 | 1.69 | 1.14 | 1.16 | 0.61 | 0.62 | 0.43 | 21.0 |

TABLE IV

MONOSACCHARIDES IN WATER EXTRACTS OF PLANTS FROM THE FIRST AND SECOND HARVESTS OF BIRD'S-FOOT TREFOIL AS DETERMINED BY GAS-LIQUID CHROMATOGRAPHY

| Sample | Water Extract Hydrolyzed | Water Extract Unhydrolyzed |
|---|---|---|
| Harvest | Second % | Second % |
| α-Erythrose | 11.92 | 16.41 |
| β-Erythrose | 15.02 | 6.56 |
| α-Arabinose | - | - |
| β-Arabinose | - | - |
| s-Arabinose | | |
| α-Xylose | 0.71 | trace |
| β-Xylose | trace | - |
| α-Fructose | 4.81 | 3.49 |
| β-Fructose | 2.04 | 2.08 |
| α-Mannose | 20.09 | 21.57 |
| β-Mannose | | |
| α-Glucose | 3.34 | 4.60 |
| β-Glucose | 2.75 | 2.24 |
| Galactose | trace | trace |
| i-inositol | 0.37 | 0.63 |
| Total/g of dry tissue | 61.05 mg | 57.58 mg |

## SUMMARY

Gas-liquid chromatographic studies were made for both free and combined sugars in the leguminous plant, bird's-foot trefoil (Lotus corniculatus L.). The result of the investigation indicated the following:

1. Erythrose was present in considerable amounts as a free-sugar.
2. Both the α and β forms of mannose, fructose, glucose and galactose were present and comprised the main portions of free sugars in the forage.
3. The free-sugars erythrose, mannose, fructose, glucose and galactose exist probably in ratios of 9:25:5:7:1.
4. No sucrose was detected.
5. Neither starch nor fructosan was present.
6. Apparently, some water insoluble pentosans (araban or xylan) or some hemicellulose (arabinoxylan or arabinogalactan) exist in this forage.
7. Some polysaccharides such as mannan galactan, galactomannan exist in this plant as reserve food. Bird's-foot trefoil appears to contain different polysaccharides than do the grasses.
8. A considerable amount of i-inositol occurred in 80 percent ethanolic extracts, water, and acid extracts of the tissue.
9. Quantities of total available carbohydrates, as deter-

mined by four different methods of extraction varied. The order of magnitude for the amounts extracted are as follows: 0.8 N sulfuric acid > 0.2 N sulfuric acid > water ≈ 80 percent ethanol.

10. The plant tissues of the second harvest (July 16, 1962) contained almost 33 percent more free-sugars and total carbohydrates than did the plants from the first harvest (July 4, 1962).

11. Both samples of the first and second harvests contained the same kinds of sugars and the ratio of sugars in both harvests were almost identical in extracts of the same solvent.

12. The branched chain five-carbon sugar apiose, which was detected in clover and some other perennial legumes, was not detected in bird's-foot trefoil.

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