Carbohydrates are the primary source of reserve energy stored in the vegetative organs of biennial and perennial forage plants. Reserves of available carbohydrates are essential to survival and to the production of plant tissues during periods when carbohydrate utilization exceeds photosynthetic activity.

Total available carbohydrate levels and trends in storage organs are useful in indicating the periods of storage and usage and are useful in evaluating the potential of plants for regrowth and production following defoliation. A number of methods have been used to extract total available carbohydrates from forage plant tissue. However, the method of extraction used will influence greatly the values obtained because forages vary in the type of carbohydrate stored.

The common perennial and biennial forage legumes, such as alfalfa, red clover, white clover, and sweetclover, are characterized by the accumulation of sucrose and starch (3, 9, 11). Extraction of total available carbohydrates from these species has been accomplished largely by the takadiastase enzyme method developed by Weinmann (16, 17) or with acid solutions (8, 10). The common perennial forage grasses belong to 2 groups according to the type of reserve carbohydrate stored. Grasses native to tropical and subtropical latitudes, such as bermudagrass, bahiagrass, and dallisgrass, are characterized by the accumulation of sucrose and starch. Extraction of total available carbohydrates has been done as described above for legumes (17, 18). Grasses native to temperate latitudes, such as timothy and orchardgrass, accumulate sucrose and fructosan (1, 4, 7, 12, 13). Extraction of total available carbohydrates has been accomplished by acid hydrolysis (8) or with water (1, 14, 15) since fructosan is soluble in water. Starch, however, is largely water-insoluble.

This study was initiated to compare enzyme, dilute acid, and water extraction methods for the estimation of total available carbohydrates in forage plant tissue. Timothy stem bases and alfalfa roots were used as the test tissues. Timothy stores the largest proportion of its reserve carbohydrates as fructosan in the stem bases (4, 7). Alfalfa stores most of its reserve carbohydrates as starch in the roots (3).

**Materials and Methods**

Selected samples of timothy (Phleum pratense L.) stem bases and of alfalfa (Medicago sativa L.) roots were used. Timothy samples were from a season of growth under 2 harvests for hay, June 27 and August 29 (8). Alfalfa samples were from the early part of a season under a hay harvest on June 3 (10).

The fresh tissue was dried in a forced-draft oven at 100°C for one hour and collected at 70°C. The dried tissue was ground to pass through a 40-mesh sieve, placed in glass bottles, and dried at 70°C to constant weight. The bottles were tightly capped, and stored for analysis.

Enzyme extraction was accomplished according to the method of Weinmann (16) using the enzyme preparation and modifications of Lindahl et al (6). Essentially the sample was mixed with water and refluxed in a boiling water bath to gelatinize any starch. The mixture was then incubated at 38°C for 44 hours with a buffered takadiastase (Clarase) enzyme preparation. Proteins were removed with neutral lead acetate and the excess lead was removed with potassium oxalate. A 10 ml aliquot of the filtered solution was hydrolyzed with 0.5 ml of 25% HCl for 30 minutes in a boiling water bath. The solution was cooled, neutralized with NaOH solution, and diluted to volume with distilled water.

Acid extractions were made with solutions ranging from 0.2 x to 0.8 x H₂SO₄. Results are reported only for the 0.2 x and 0.8 x extractions. Each sample was placed in a 200 ml round-bottom flask with 50 ml of acid, and refluxed for 60 minutes in a boiling water bath. The hot solution was filtered through Whatman No. 42 filter paper. Proteins were not removed. The filtrate was cooled, neutralized with NaOH solution, and diluted to volume with distilled water.

Water extractions were made with hot and cold water. Each sample was placed in a 200 ml round-bottom flask with 50 ml of distilled water. The hot-water samples were attached to reflux condensers and refluxed 1 hour in a boiling water bath. The cold-water samples were stoppered and placed on a Burrill shaker for 1 hour at room temperature. The solutions were filtered through Whatman No. 42 filter paper, and the residue was washed with 25 ml of distilled water. Proteins were not removed. One ml of 25% HCl was added and the solutions were hydrolyzed for 30 minutes at 75°C. The solutions were cooled, neutralized with NaOH solution, and diluted to volume with distilled water.

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2 Published with approval of the Director of the Wisconsin Agricultural Experiment Station.
3 Total available carbohydrates in this paper are the carbohydrates that are readily available as a source of energy to the plant. Included are the sugars, dextrin, starch, and fructosan but not structural carbohydrates, such as hemicellulose and cellulose.
Carbohydrate content of the above solutions obtained by the various extraction procedures was determined by analyzing for reducing power with the Shaffer-Somogyi copper-iodometric titration method outlined by Heinz and Murneek (5) using fructose standard solutions for the timothy and glucose standards for the alfalfa. The results were expressed as per cent total available carbohydrates on a dry weight basis (70°).

Results

The enzyme method of extraction for total available carbohydrates was used as the check method. Starch in this method is extracted and partially hydrolyzed by the takadiastase enzyme preparation. Soluble sugars and fructosan are extracted during incubation with the enzyme because of their solubility in water. Complete hydrolysis of the extracted carbohydrates to reducing sugars is accomplished subsequently with acid, and the reducing power of the neutralized hydrolysate is determined. Thus, both starch and fructosan are a part of the total available carbohydrate content.

The trends of total available carbohydrates in the 15 timothy stem base samples are shown graphically in figure 1, and in the 10 alfalfa root samples in figure 2. The extraction method with values most nearly like those of the enzyme method was extraction with 0.2 N H₂SO₄.

Each method of extraction, except for the water extractions of alfalfa roots, provided results that showed the same trend and indicated the same periods of increase and decrease of carbohydrates. The principal difference among extraction methods was the level of values obtained. In the case of the water extractions of the alfalfa roots, the values did not follow exactly the trend as established by the acid and enzyme extractions. This was probably because only free sugars were being extracted and that the insoluble starch remained in the residue.

Discussion

Extraction with 0.2 N H₂SO₄ gave carbohydrate values that were very similar to those of the enzyme extraction in both timothy and alfalfa. The acid values averaged only 0.4% higher for the timothy and only 0.5% higher for the alfalfa than the enzyme values. Extraction with 0.8 N H₂SO₄ gave values that were considerably higher than with 0.2 N H₂SO₄. The stronger acid solution was probably extracting structural carbohydrates, such as the hemicelluloses.

Much less time was involved during extraction with 0.2 N H₂SO₄ than with the enzyme method even though the carbohydrate values obtained were similar. Acid extraction required about 8 hours for a complete analysis compared with 12 hours working time plus 44 hours incubation for the enzyme method.

The water extractions gave lower values than the enzyme method in both timothy and alfalfa. With timothy, the water extractions were removing both the free sugars and fructosan. The values were approximately the same as the total values obtained by Okajima and Smith (7) with these same samples after extraction of the sugars with 80% ethanol and of the fructosan with cold water. The differences in values between the water extractions and enzyme extractions of timothy were due probably to insoluble carbohydrates, possibly starch, that were not being removed with water but were removed under the conditions of the enzyme method. The values averaged 5.0% lower for hot water and 6.9% lower for cold water than the values from the enzyme method. Okajima and Smith (7) obtained similar values (averaging 2.7%) with these same samples when the residue following extraction of the sugars (with 80% ethanol) and of fructosan (with cold water) was treated with the enzyme method of extraction.

The water extractions of the alfalfa removed only the free sugars. The values were approximately the same as those obtained with the 80% ethanol extraction (unpublished data). The insoluble carbohydrates, including starch, remained in the residue.

Slightly higher values were obtained consistently with hot water than with cold water extraction as shown clearly in figures 1 and 2. The average dif-

![Fig. 1. Per cent total available carbohydrates in timothy stem bases with various methods of extraction.](image-url)

Fig. 1. Per cent total available carbohydrates in timothy stem bases with various methods of extraction. The difference was 1.9% for timothy and 0.8% for alfalfa. The difference may be due to the greater solubility of a relatively insoluble carbohydrate, possibly gelatinized starch, in hot water.

The determination of total available carbohydrates by analyzing for water-soluble carbohydrates (free sugars and fructosan) will give results similar to those obtained from the enzyme method only when starch is absent in the tissue or when starch is present only in very small amounts. The problem is that fractionations have been made on some legumes and on only a few grasses so that it is not known to what extent they store starch and/or fructosan. If starch is stored in any appreciable amounts, the results of
the enzyme extraction method and of water extraction will not be comparable. Dodd and Hopkins (2) recently reported as much as 11% starch and 19% fructosan in the roots, rhizomes, and crowns of blue grama grass (Bouteloua gracilis) in the fall growth. The best method of extraction in determining total available carbohydrates would appear to be the enzyme or 0.2N H2SO4 extraction methods because the conditions of both methods will include the sugars, starch, and fructosan in the final carbohydrate values.

Summary

A comparison was made of enzyme, dilute acid, and water extraction methods for the estimation of total available carbohydrates in timothy stem bases and alfalfa roots.

Extraction with takadiastase enzyme and 0.2N H2SO4 methods gave carbohydrate values that were very similar, and the conditions of both methods are such as to include the sugars, starch, and fructosan in the final carbohydrate values. Extraction with a stronger acid solution gave higher values probably because structural carbohydrates were being hydrolyzed. Much less total analytical time was involved in the acid extractions than with the enzyme; something on the order of 8 hours compared with 56 hours.

Carbohydrate values were slightly higher with hot water than with cold water extraction.

The use of water extraction to estimate total available carbohydrates gave values of the same physiological significance as the enzyme method only when starch was absent or present in very small amounts.

Literature Cited