Comparison of Two Methods for the Routine Extraction and Quantification of Non-Structural Carbohydrates in Tree Tissues

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ABSTRACT

Rooting success of cuttings for vegetative propagation is largely dependent on the reserve of carbohydrates in the plant tissue. In order to predict rooting potential of cuttings, analysis of their non-structural carbohydrate content is necessary. Few methods for simple extraction and quantification of storage carbohydrates exist. Widely used with large sample quantities is the anthrone method which, however, poses safety and environmental hazards through the use of concentrated acids. An enzymatic test specific to glucose was compared with the anthrone method. Shoot and root samples of *Melia volkensii*, an indigenous tree in Kenya, were used to compare the methods. Whereas the enzymatic test proved highly reliable for free glucose, it was not useful for the analysis of glucose derived after hydrolysis of starch with perchloric acid. For starch analysis, the iodine/iodide colour reaction was found to be acceptable. Taking the limitation of the anthrone method into account, it was found the most useful for determination of total soluble sugars within plant samples.

KEY WORDS: anthrone, carbohydrates, glucose-6-dehydrogenase, iodine, sugars, starch, *Melia volkensii*, vegetative, propagation

1.0 INTRODUCTION

It has been reported that one of the most important factors in rooting success of stem and root cuttings for vegetative propagation is the carbohydrate reserve in the cuttings which enable them to metabolise until a functional root system has been developed (Leakey, 1983; Leakey and Coutts, 1989; Leakey and Storeton-West, 1992). Intensive studies are ongoing with a range of indigenous tree species to determine appropriate stockplant pre-treatments to raise the carbohydrate content in cuttings to allow for maximum rooting success.

Carbohydrates are the most abundant chemical constituents of plants, making up 60-90% of their dry matter. Water-soluble sugars, with a relatively simple molecular structure, are involved in intermediate metabolism and act both as substrates for the synthesis of more

complex compounds and as respiratory intermediates. More complex chain molecules are starch, the common storage form of carbohydrates, and cellulose, one of the components of cell walls and other structural elements. Starch can be enzymatically hydrolysed in plants into glucose molecules to provide energy, whereas cellulose can only be hydrolysed by a specialised group of enzymes not present in plants (Kozlowski and Pallardy, 1997).

The aim of this study was to identify a method which allows for the routine determination of non-structural metabolisable carbohydrates with simple equipment in order to enable local laboratories to perform high-quality and reproducible analyses. Analysing sample material of the Kenyan indigenous and important tree species *Melia volkensli* at the ICRAF Research Facilities at Machakos, Kenya, we adapted and tested two published methods, the 'anthrone' method (Dreywood, 1946, cited in Yemm and Willis, 1954) which determines total monosaccharide sugars in plant tissue, and an enzymatic method specific to glucose, developed for medicinal purposes (Chaplin, 1994). These two methods were selected after an extensive review of other potential methods, such as High Performance Liquid Chromatography (Sudin and Shaarl, 1988) and paper chromatography (Yemm and Willis, 1954; Southgate, 1969) which were rejected because they involve more demanding technology.

The anthrone reagent was first introduced for sugar determination in plant samples by Dreywood in 1946 (cited in Yemm and Willis, 1954). It reacts with the most common monosaccharides, giving a blue colour which can be measured colorimetrically. A reaction time of 7.5-10 minutes at 100°C has been adopted for the routine analysis with large samples. Under these conditions, glucose and fructose which together usually make the bulk of hexoses in plant tissue, give a fairly accurate estimate of total sugars. (Scott and Melvin, 1953; Yemm and Willis, 1954; Deriaz, 1961; Jermyn, 1975; Hassid and Neufeld, 1979; Deluca and Keeney, 1993). Analysis of total sugars involves two extractions; first, the extraction of soluble sugars in hot ethanol and then hydrolysis of starch and other glucose polymers from the same sample with perchloric acid into glucose residues. The reagent is not specific for glucose. A major drawback of this method is the use of concentrated sulphuric acid which is highly corrosive, and the need for large sample volumes.

Enzymatic methods for the determination of sugars are highly specific and allow accurate determination of single components. Enzymatic starch hydrolysis avoids the degradation of structural tissues and has been employed extensively (Smith, 1969, 1971; Dekker and Richards, 1971; Haissig and Dickson, 1979).

2.0 MATERIALS AND METHODS

All chemicals used were analytical grade and obtained from BDH, UK, unless otherwise specified. Deionised water was used for all reagent preparations.

2.1 Pre-treatment of samples

Samples of fresh stem or root tissue of Melia volkensii were harvested, immediately frozen in liquid nitrogen to deactivate enzymes, and dried in a forced-draught oven at 65-70°C to a constant weight. Dried samples were then ground using a ball mill to pass a 0.5-mm screen and stored in sealed glass vials at room temperature.

2.2 The anthrone method (after Jermyn, 1975)

2.2.1 Extraction of soluble sugars:

Finely ground sample (0.02 g) was weighed into a centrifuge tube and 0.5 ml water added. 2.5 ml of hot 80% ethanol was then added and the solution mixed well using a vortex mixer and left to stand for 5 minutes after which it was centrifuged at 5,000 rpm for 5 minutes. The supernatant was decanted into 15-ml test tubes, 0.5 ml water added to the residue and the whole process repeated. The two extractions were combined and 6 ml water added to the combined extractions.

2.2.2 Hydrolysis and extraction of starch:

To the pellet remaining after the extraction of soluble sugars, 0.5 ml water was added. The tubes were put on ice and 0.65 ml of 52% perchloric acid was added. The samples were then well stirred using a glass rod and allowed to stand on ice for 20 minutes with occasional stirring. After removing from the ice, the reaction was stopped by adding 2 ml water, the solution mixed well and centrifuged at 5,000 rpm for 5 minutes. The supematant was decanted into 15-ml test tubes, 0.5 ml water added again to the pellet and the whole process repeated. The two extractions were combined and 6 ml water added to the combined extractions.

2.2.3 Analysis:

Both extractions were assayed for soluble sugars. 50 µl of each samples and standards were pipetted into 50 ml test tubes. The tubes were put on ice and 2.5 ml water added. 5 ml of anthrone reagent (1 g anthrone in 500 ml refrigerated concentrated sulphuric acid, mixed fresh every two days) was carefully added to each tube using a dispenser, and vortexed immediately.

The samples were kept on ice until all were prepared, then heated for 7.5 minutes in a boiling water-bath (95°C at an altitude of 1,800 masl). They were then immediately cooled on ice to room temperature, mixed well and left to stand for 15 - 20 minutes before reading the absorbance in a spectrophotometer at 630 nm.

2.3 The hexokinase/glucose dehydrogenase assay (after Chaplin, 1994)

2.3.1 Preparation of reagents:

Buffer solution: 6 g triethanolamine hydrochloride and 0. 11g magnesium sulphate ($MgS0_4$)7 H_20 (or 0.052g $MgS0_4$) were dissolved in 80 ml water. pH was adjusted to 7.6 with 5 M sodium hydroxide (NaOH), then the solution made up to 100 ml with water. **Nicotinamide adenine dinucleotide phosphate (NADP) solution:** 0.043 g NADP was dissolved in 10 ml water. **Adenosine triphosphate (ATP) solution:** 0.22 g ATP disodium salt hydrate plus 0.22 g sodium hydrogen carbonate ($NaHCO_3$) were dissolved in 10 ml water. **Enzyme solution:** 0.100ml hexokinase (HK) (Fluka,140 U/Mg), 0.250 ml glucose-6-phosphate dehydrogenase (G6PDH) (Fluka, 283 U/mg protein) and 9.650 ml ammonium sulphate solution (6 g in 10 ml water) were mixed thoroughly and kept in the refrigerator.

2.3.2 Extraction:

The same process as described above was used.

2.3.3 Analysis:

Samples and standard solutions (100 µl of each) were added to a mixture containing 1,000 µl buffer solution, 100 µl NADP solution and 100 µI ATP solution and mixed well. The reaction was started with 100 µl of enzyme solution, containing 0.14 units HK and 0.07 units G6PDH. After further mixing, the samples were incubated for 60 minutes at 37°C. After incubation, the samples were cooled and their absorbance readings determined at 340 nm.

2.4 The iodine/iodide starch determination

2.4.1 Extraction:

The pellet that remained after extraction of soluble sugars with 80% ethanol was dried and then a sub-sample of 0.01 g washed in about 2 ml acetone (GPR grade). It was then centrifuged at 10,000 rpm for 5 minutes and excess acetone decanted. The remainder was dried at 60°C until all acetone had evaporated. To the dry pellet, and to an equivalent amount of

potato starch for the preparation of standard solution (200µ starch/1.2 ml), 200 µl of 1. 55 M KOH was added, the solution mixed well and boiled for 10 minutes in a water bath. 800 µl of 0.25M acetic acid was added, mixed well then centrifuged for 5 minutes at 1 0,000 rpm. The supernatant was used for analysis.

2.4.2 Analysis:

200 µl of both samples and standards were pipetted into cuvettes. To each, 1000 µl of iodine reagent (0.025 ml iodide solution (2.6 g potassium iodine and 0.26 g iodine in 10 ml of water) and 6.5 ml saturated calcium chloride (75 g $CaCl_2 2H_20$ in 100 ml water), prepared fresh daily) was added. The solutions were mixed well and the absorbance readings taken immediately at 620 nm.

3.0 METHOD DEVELOPMENT AND DISCUSSION

For the adaptation of the enzymatic method, the optimal enzyme concentration was established. To samples containing 40µg glucose, different amounts of HK and G6PDH were added and the reaction monitored over a period of 75 minutes (Figure 1).

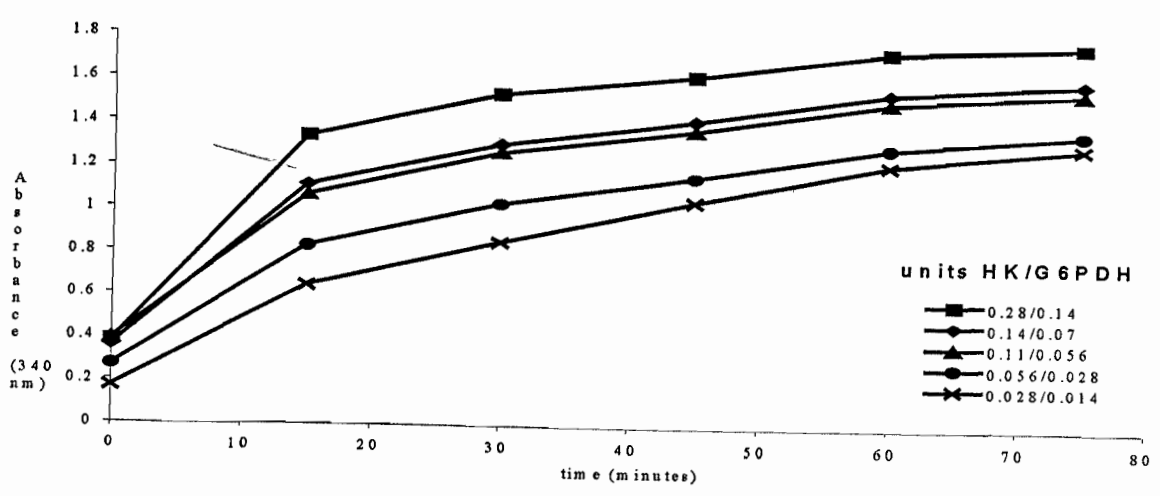

Fig. 1 Effect of enzyme concentrations and incubation at 37°C on digestion mg glucose

After a sharp increase in absorbance within 15 minutes, during which 75% of the maximum absorbance was reached, the reaction slowed considerably. Whereas the lower concentrations (0.56 units HK and 0.026 units G6PDG per 1.4 ml sample) and (0.028 units HK/0.014 units G6PDH)) showed a clear lack of enzymes to convert the glucose fully into glucose-6-

phosphogluconate, the higher concentrations show nearly complete conversion. Allowing for a maximum 10% error, we decided to use 0. 14 units HK/0.07 units G6PDH for future analysis. This enzyme concentration reached 90% of the maximum of the highest concentration (0.28 units HK/0.14 units G6PDH) and was statistically not different from the higher concentration (SD=0.1). 60 minutes incubation time was considered sufficient as over 95% of the reaction had taken place within this time.

To establish whether hot 80% ethanol would disturb the enzymatic reaction, we compared both water and 80% ethanol extracts using glucose standards (Figure 2). Ethanol in high concentration is toxic to most enzyme systems. However, using small amounts for the extractions, its concentration in the samples was sufficiently diluted (to ca 35%) to not have an adverse effect on the enzymatic activity. Since it is a better extractant for soluble sugars it was used in the routine extractions.

Although we established that the enzymatic glucose assay worked well with the alcohol-extracted fraction of soluble sugars, we could not analyse the hydrolysed starch fraction. There was evidence of very serious interference or inactivation of the enzyme in the second extraction, even after neutralization of the extract with KOH (Table 1, columns D and E).

We decided to use the iodine/iodide test for quantification of starch to analyse the second fraction of non-structural carbohydrates in plant samples. Although the test is generally used for semi-quantitative analyses we adapted it to give reliable quantitative results.

The iodine/iodide method has several drawbacks: (1) colour sensitivity of iodine decreases with increasing temperature of solution (at 50°C, it is ten times less sensitive than at 25°C); (2) the sensitivity decreases upon addition of solvents such as ethanol with a complete lack of colour development in solutions containing 50% ethanol or more, making thorough washing of the extracts with acetone necessary; (3) iodine cannot be used in a strongly acid medium as hydrolysis of the starch occurs, making neutralisation necessary.

Table 1 Comparison of the methods tested with tissue samples of *Melia volkensii*

Sample ID	Sugar fraction A	Sugar fraction B	Starch fraction C	Starch fraction D	Starch fraction E	Starch fraction F
	Anthrone Method (hexoses)	Enzymatic method (glucose)	Anthrone method (glucose derived from starch hydrolysis)	Enzymatic method (glucose derived from starch hydrolysis	Enzymatic method (glucose derived from starch hydrolysis; sample neutralised)	Iodine/ iodide method (starch
7/7	277.29 (15.7)	13.55 (5.1)	258.45 (10.9)	24.54	23.73	315.11 (2.1)
7/10	349.20 (20.5)	6.36 (0.3)	277.62 (20.5)	29.14	23.90	316.79 (8.1)
15/1	267.08 (12.2)	3.07 (1.0)	470.34 (3.7)	24.83	22.39	395.77 (4.1)
33/1	261.90 (19.6)	11.67 (4.4)	534.34 (1.2)	24.30	23.49	635.88 (8.8)
33/4	314.96 (23.0)	9.17 (2.1)	494.80 (8.5)	24.30	23.49	570.00 (6.5)

The free sugar fraction was extracted with 80% ethanol, the starch fraction with 52% perchloric acid, apart from iodine/iodide method which tests directly for starch. Values are in mg/g DM: column A: free monosaccharides; columns B-E: glucose, column F: starch. Means of 2 samples, columns D/e one sample (SD in brackets).

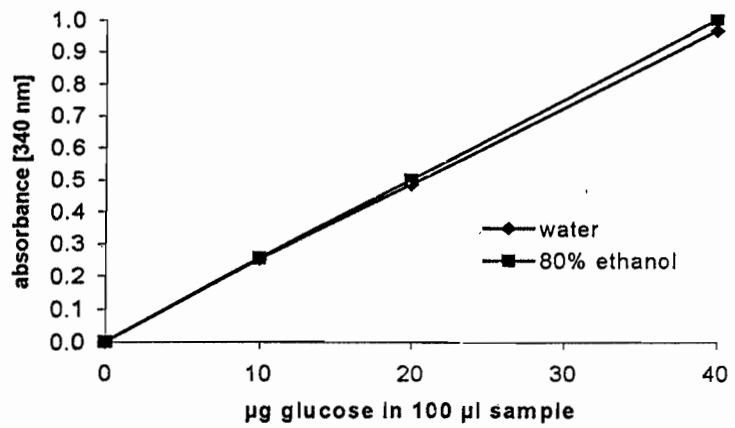

Fig. 2 Effect of water and 80% ethanol as extractants of glucose on enzymatic method

However, after appropriate sample preparation, the colour development of the reagent is sufficiently linear between 0 and 80 µg starch/1.2 ml sample (Figure 3).

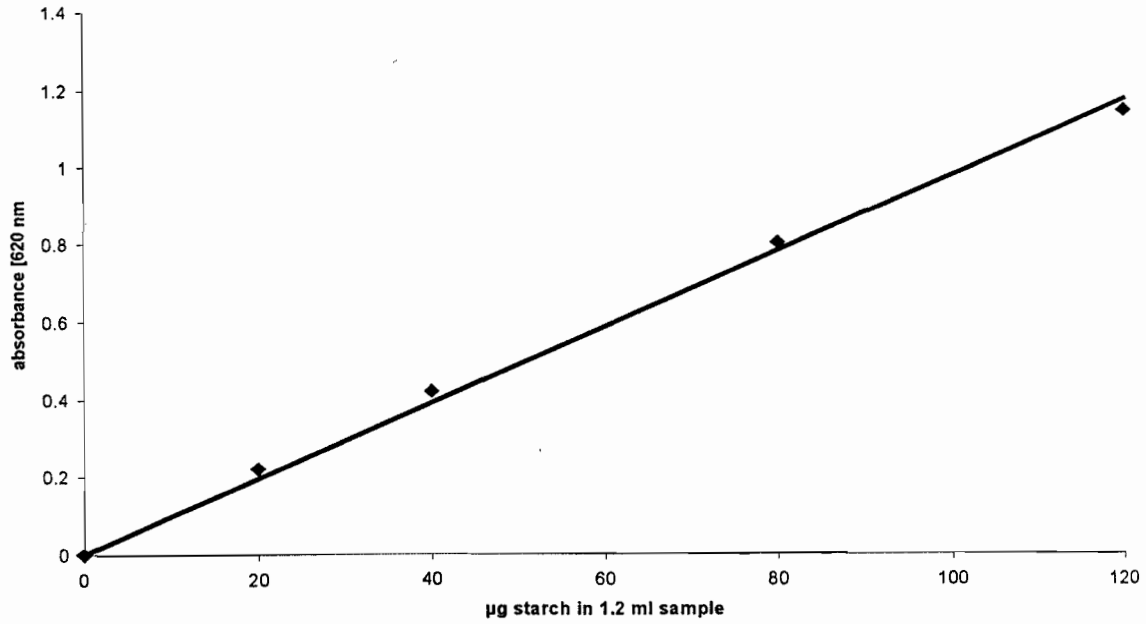

Fig. 3 Absorbance readings [620nm] of standard starch samples in iodine/iodide extraction (details see text)

4.0 CONCLUSIONS

Comparing the results of the anthrone method with the enzymatic method, the former gave substantially higher values for the soluble sugars fraction (Table 1) indicating that transport sugars in *Melia volkensii* are composed to a significant extent by sugars other than glucose. However, we found the enzymatic method reliable and highly effective (96% recovery), whereas obtaining reliable data using the anthrone method depended to a large extent on the precision of the analyst. Problems included an unstable reagent which resulted in high blanks and variable replicates. In addition, the anthrone reagent itself darkens on heating, and the temperature required for accurate reaction (Jennyn, 1975) cannot be reached in altitudes higher than 700 masl. Heating the samples at the correct temperature for the exact amount of time is crucial in obtaining reproducible results.

The drawback of the enzymatic method, which was unsuitable to analyse the starch fraction, led to the adoption of a combined approach: glucose-specific enzymatic tests if desirable, combined with iodine/iodide starch determination for accurate results. In cases were total soluble sugars are of interest, the anthrone method is still the most appropriate method, taking its limitations into account.

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