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COMPARISON OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND ENZYMATIC ANALYSIS OF SOLUBLE CARBOHYDRATES IN LOBLOLLY PINE¹

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ABSTRACT: Foliar tissue was collected from a field study designed to test impacts of atmospheric pollutants on loblolly pine (*Pinus taeda* L.) seedlings. Standard enzymatic (ENZ) and high performance liquid chromatography (HPLC) methods were used to analyze the tissue for soluble sugars. A comparison of the methods revealed no significant differences in accuracy or in detection of treatment differences, but did find the HPLC results had a greater within-method variability, thus lowering method precision. This variability may be reduced by consistent maintenance and monitoring of sugar detection. If both methods are performed with equal care, soluble sugar values will be comparable.

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INTRODUCTION

Carbohydrate production and mobilization influence the ability of plants to regulate functions such as the acquisition and transport of nutrients (2), maintenance and expansion of roots (1,5,6) and resistance to environmental stresses (7). Quantitative measures of available carbohydrates (i.e., hexose, sucrose, and starch) are useful in gaining a fuller understanding of plant physiological processes. Available carbohydrate levels may be analyzed by various methods that differ in their complexity and sensitivity to detect and quantify the individual sugars. The method chosen will depend on the objectives of the study, the accuracy required, and the available resources.

The two techniques compared in this paper are an enzymatic (ENZ) assay and high performance liquid chromatography (HPLC). In the ENZ method, enzymatic conversion of fructose, sucrose and starch to glucose is measured indirectly by assaying conversion of nicotinamide adenine dinucleotide phosphate (NADP) to NADP(H). The amount of conversion is directly proportional to the glucose content of the sample (3). The detection of glucose units is by a colorimetric reaction that is measured by spectrophotometry. Detection range for this method is 30 to 300 ppb of glucose equivalents (3). The HPLC system employed in this comparison has an ion chromatograph with a pulsed amperometric detector, and extracted carbohydrates are identified after oxidation at a gold electrode (8). Detection limits for this method have been reported as low as 30 ppb for monosaccharides (i.e., glucose, fructose) and 100 ppb for oligosaccharides (i.e., sucrose, trehalose) (8).

The major advantage of each method is the high level of specificity in detecting individual sugars. For the purposes of our studies, the ENZ method has been adapted to identify hexose (fructose + glucose combination) and sucrose. Other species of sugars may be measured through the addition of selective enzymes and minor procedural modifications. The HPLC method of analysis differentiates simple monosaccharide mixtures and oligosaccharides. These may include: glucose, fructose, sucrose, trehalose, raffinose, and maltose. Additional

sugar peaks can be detected by proper manipulation of eluent strength and flow speed, with quantification dependent on accurate identification of each sugar.

The ENZ method for soluble sugar analysis has been thoroughly tested on loblolly pine tissue and is deemed to be reliable and fast (Schoeneberger, unpublished data). The processing time is 4-5 days for a batch of 96 samples, including duplicates and standards. The required standard laboratory equipment (centrifuges, concentrator, and spectrophotometer) and basic technical knowledge allow this method to be readily adapted to most labs. However, the ENZ method is labor intensive and does necessitate the use of small quantities of lead acetate for removal of phenolics and other interfering compounds found in pine tissue.

The HPLC method has also been tested extensively at our laboratory. Like the ENZ technique, it has a processing time of 4-5 days for a batch of 96 samples. Speed of sample processing varies depending upon the number and species of sugars being analyzed. Automation capabilities of the HPLC allow analyses to be conducted overnight, thus dramatically reducing labor requirements. The HPLC method does require more elaborate and expensive equipment and a higher level of operator expertise than the alternative ENZ method.

The primary objective of this study was to compare the accuracy and precision of the two techniques with regard to quantified soluble sugar concentrations. A secondary objective was to determine whether the method of available carbohydrate analysis affected statistical detection of treatment differences. This consideration is important for laboratories involved in cooperative studies requiring data transfer and comparison.

METHODS AND MATERIALS

Plant material for the comparison was loblolly pine foliage collected from a field study designed to test the effects of ozone and acid deposition on loblolly pine seedlings from known open-pollinated families (4). Detection of any treatment differences from the field study would be compared between carbohydrate methods. The available soluble carbohydrates targeted for analysis in the field

study were sucrose and hexoses, which are a combination of fructose and glucose. Comparison of the two methods (ENZ and HPLC) was therefore limited to these sugars. Samples were collected and kept separate by block (micro-site differences), chamber (ozone treatments), family-within-chamber, and flush-within-family. Needle fascicles were collected during full-sun periods, quick frozen with dry ice, and freeze-dried in preparation for analysis (10). Ground tissue was stored at 0°C pending analysis.

Available carbohydrates were extracted according to the procedure outlined by Schoeneberger et al. (1992). Approximately 0.025 g of ground tissue was extracted three times with 10 ml of 80% ethyl alcohol (EtOH) to produce a total volume of 30 ml supernatant. This supernatant was stored at $\leq 0^\circ\text{C}$ prior to the method analyses.

For the ENZ technique, a 1 ml aliquot of supernatant was evaporated to dryness in a vacuum centrifuge and resuspended to 4 ml in a solution of deionized water, lead acetate, sodium carbonate and hydrochloric acid. A 0.5 ml aliquot of this centrifuge suspension was used for the ENZ assay (10). The prepared samples (a total volume of 2 ml) were analyzed for light adsorption at a wavelength of 340 nm on a spectrophotometer. The final hexose and sucrose values were calculated as glucose equivalents in mg/g according to formulations listed in Schoeneberger et al. (1992).

The HPLC method required a smaller aliquot (1 ml) of supernatant. This volume was also evaporated to dryness, but was resuspended to 2 ml in deionized water. One ml of this new solution was further diluted with 4 ml of deionized water for a final volume of 5 ml. This solution was then processed through a Dionex ion chromatograph Series 4000i with ion pac carbohydrate column HPIC AS6. The eluent was 0.035 M sodium hydroxide solution, and the electrode voltages were +0.07, +0.60 and -0.60 volts. Peak occurrence was recorded as mg of sugar per 100 ml of solution. Concentrations of glucose, fructose, and sucrose (mg per gram of dried tissue) were calculated with the following equation:

$$A \times B/C \times D/E \times F/G = \text{mg/g} \quad (1)$$

where: A = concentration value from HPLC (mg/100 ml⁻¹)
 B = volume of EtOH used in the initial extractions (ml)
 C = plant tissue dry weight (g)
 D = resuspended volume (ml)
 E = evaporated volume (ml)
 F = final solution volume for HPLC (ml)
 G = aliquot used in making up final solution for HPLC (ml)

Concentrations of the hexose sugars, glucose and fructose, from the HPLC analyses were summed for comparison with the ENZ hexose values. Sucrose values estimated by the two methods were compared directly. Total soluble sugars were calculated as the sum of hexose and sucrose values. For quality control (QC) purposes, 15% of the samples was duplicated and a standard tissue was analyzed with each set of 20 samples. The standard tissue was a bulk sample of loblolly pine seedling roots collected, processed, and stored as recommended by Schoeneberger et al. (1992).

Statistical analyses of the QC data were performed using PC Statistical Analysis Systems (SAS) procedures for univariate and general linear models (9). Coefficients of variation (CV) were computed to assess method precision and accuracy. To test the impact of carbohydrate method on the detection of treatment differences in the field study, an analysis of variance (ANOVA) (9) by carbohydrate method and flush was done. Sample data were examined for normality and homogeneity of variance using F_{\max} test ($F = s^2_{\max}/s^2_{\min}$) (11) and ANOVA, and data were weighted as deemed necessary.

RESULTS AND DISCUSSION

Method Precision: CVs reveal both similarities and differences in the precision of the two carbohydrate methods. Precisions of the methods differed significantly at the 0.05 level for hexose and at the 0.10 level for total soluble sugars (Table 1). CVs for hexose and total soluble sugars were below the 5% level for the ENZ

TABLE 1. Quality control data for method precision estimates showing mean sugar concentration and range of concentrations in mg/g glucose equivalents, and mean coefficients of variation (CV) and range of CV in percentile.

parameter	Concentration of glucose equivalents			Coefficient of variation		
	Mean mg/g	Range min max mg/g	Mean %	min %	max %	Mean %
<u>Zymatic Method</u>						
Hexose	88.7	61.1 108.3	1.7	0.1	4.5	
Sucrose	4.4	0.2 11.7	17.7	1.3	95.2	
Total soluble sugars	93.1	64.9 112.8	1.6	0.2	4.6	
<u>LC Method</u>						
Hexose	84.4	56.2 122.3	3.7*	0.3	15.7	
Sucrose	4.7	0.0 13.9	26.1	0.6	141.4	
Total soluble sugars	89.1	59.0 122.3	3.3 ⁺	0.1	12.4	

*: an coefficients of variation for the two methods of sugar analysis differ significantly;
 †: P ≤ 0.05, and †+ = P ≤ 0.10.

method, with values ranging from 0.1 to 4.5% for hexose and 0.2 to 4.6% for total soluble sugars. For the HPLC technique, CVs ranged from 0.3 to 15.7% for hexose and 0.1 to 12.4% for total sugars (Table 1).

No significant differences were evident for sucrose analyses between the methods. Coefficients of variation ranged from 1.3 to 95.2% in the ENZ data and from 0.6 to 141.4% in the HPLC data. These large CV values were due to the low levels of sucrose in the majority of samples. Sucrose values from the ENZ analysis ranged from 0.2 to 11.7 mg/g, and for the HPLC analysis sucrose was quantified from 0.0 to 13.9 mg/g (Table 1). Prior testing of the ENZ method had shown that detection of sucrose levels below 10 mg/g was not reliable. Data from this study indicate that the same limitation exists with the HPLC method, but modifications of eluent concentration and flow rate may improve detection performance at these low sucrose levels.

Method Accuracy: To compare the accuracies of the two methods, carbohydrate standard tissue (CST) values were estimated by the ENZ and HPLC methods with CST values obtained over four separate dates in 1990. The CST analyzed with each field sample set had been thoroughly tested using the ENZ method of analysis. No significant differences were found between CV values of the two methods and 1990 values for hexose, sucrose, or total soluble sugars. Hexose CVs ranged from 4.1 to 6.8%, sucrose from 4.1 to 35.5%, and total soluble sugars from 0.8 to 6.9%. CVs were elevated for sucrose, with the majority (86%) of samples having sucrose levels below 10 mg/g.

Detection of Treatment Differences: Comparison of the sample variances revealed differences of within-method variability. The ratio of HPLC to ENZ mean squares for error A were at least 1.53 times higher for total soluble sugars and as much as 12.65 times higher for sucrose in flush 2 (Table 2). The HPLC method consistently had the greater within-method variability for all parameters and across all flushes. Due to these differences in variability, an ANOVA was done with weighted data using each method's error variance (weight = $1/s_e^2$). No significant differences were detected for carbohydrate method, and only in flush

TABLE 2. Estimates of within-method variability for HPLC and ENZ soluble sugar analyses by flush and error using Fmax test for ratios of mean squares.

Parameter	Error A	Error B
-----ratio HPLC:ENZ mean squares-----		
<u>Flush 1</u>		
Hexose	2.49	3.69*
Sucrose	5.55*	4.72*
Total sugars	1.96	2.57
<u>Flush 2</u>		
Hexose	1.95	3.37
Sucrose	12.65**	3.29
Total sugars	1.53	2.15
<u>Flush 3</u>		
Hexose	6.76**	2.49
Sucrose	4.53*	2.12
Total sugars	7.19**	2.24

mean squares for the two methods of sugar analysis differ significantly; **= $P \leq 0.01$, and *= $P \leq 0.05$.

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TABLE 3. Analysis of variance of soluble sugars concentrations in loblolly pine needle tissue quantified by two different carbohydrate detection methods

Source of variation	d.f.	-----FLUSH-----								
		Hexose			Sucrose			Total soluble sugars		
		1	2	3	1	2	3	1	2	3
		-----mean squares-----								
Block (BL)	2	2.06	1.27	7.16**	0.72	0.05	1.62	2.32*	1.59	5.91**
Zone (O3)	4	2.05	4.82*	3.61	2.05	0.36	5.79	1.51	6.13**	3.74
BL x O3 (error a)	8	1.52	0.92	1.30	1.28	0.98	3.10	1.59	0.98	1.28
Family (FAM)	1	1.18	2.91	0.49	0.98	1.20	2.50	1.97	2.73	1.00
3 x FAM	4	0.68	2.96	2.85	0.81	1.40	0.62	0.61	2.94	2.43
BL x O3 x FAM error b)	10	1.58	1.55	1.71	1.30	1.37	1.57	1.55	1.49	1.79
Method (M)	1	0.04	0.28	0.43	0.35	0.00	4.48 ⁺	0.14	0.36	1.07
3 x M	4	0.16	0.24	0.54	1.75	1.21	1.67	0.09	0.17	0.44
FAM x M	1	0.38	0.18	1.55	0.08	0.62	4.61 ⁺	0.53	0.09	0.63
3 x FAM x M	4	0.11	0.56	0.24	0.15	1.07	0.29	0.07	0.48	0.25

mean squares significantly different; **= $P \leq 0.01$, *= $P \leq 0.05$, and += $P \leq 0.10$.

3 was a marginal difference for sucrose ($p < 0.056$) detected (Table 3). Ozone treatment from the field study was significantly different for flush 2 total soluble sugars ($p < 0.01$) and hexose ($p < 0.05$), but no other major effect differences were found. Overall, there was no evidence of an effect of carbohydrate method on the interpretation of the ANOVA. Thus, the results of the field experiment were the same regardless of laboratory method of sugar analysis.

CONCLUSIONS

Examination of results from accuracy data and treatment detection comparisons showed no differences between the two carbohydrate analyses. Neither method accuracy nor detection of treatment effects were statistically different. While these results indicate that the two methods are comparable for estimating soluble sugar concentrations, potential problems exist with the HPLC method. Because of the differences found in method precision, coupled with the higher within-method variability of the HPLC technique, this analysis is less precise than the ENZ procedure. HPLC variability could potentially affect the ability to detect significant differences between treatments. Since variability increases as sampling size decreases, analysis with a smaller sample set than the one used in this study might have yielded statistical differences in treatment detection.

The variability of HPLC analysis is likely due to equipment misidentification of the detection windows for individual sugars. This misidentification was more common for fructose and sucrose, which elute closely. Results of inflated ranges for both the hexose (fructose+glucose) and sucrose detection will also affect the total soluble sugar value. While the HPLC technique requires consistent standard maintenance and observation of chromatograms to ensure separate elutions for sugar identification, it does provide the opportunity to quantify multiple sugars with a single analysis. If equal care is taken in performance of both the ENZ and HPLC analyses, soluble sugar concentrations will be comparable.

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A KINETIC PARAMETER FOR EVALUATION OF THE REACTIVITY OF AGRICULTURAL LIME

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ABSTRACT: The "reactivity" and dissolution rate of an aglime in soil is not adequately described by chemical analysis involving neutralization/dissolution under harsh conditions. A method related to dissolution rate at ambient soil pH values should provide important information on aglime "reactivity". We developed a constant pH titration method, using an automated titration assembly, to achieve this aim. Fifteen aglimes, differing widely in composition and reactivity, were compared by titration at pH 4.0, 5.0 and 5.5 and noting the neutralization rate. The time, in minutes, at which 50% of an aglime's neutralizing ability (HCl-value) had been neutralized, was considered to be a useful kinetic parameter and designated $T_{1/2}$. The aglimes tested had widely different $T_{1/2}$ values even when the aglimes were comparable with regard to particle size and chemical and physical properties. The $T_{1/2}$ values were significantly correlated (negatively) with a quality parameter obtained by an acid resin suspension method.

INTRODUCTION

The estimation of the "quality" of an aglime is dependent on several measurements and parameters, some of which are unquestionably related to the dissolution kinetics thereof. Thus, in several laboratory determinations of aglime quality, reaction time is of critical importance and must be specified (1, 2, 3). Nevertheless, many of the parameters employed for aglime characterization are unrelated to dissolution and reaction rates, which obviously are, amongst others,