Phytochemistry, 1969, Vol. 8, pp. 227 to 233. Pergamon Press. Printed in England

VARIATION IN TOTAL SOLUBLE SUGARS OF CONIFER TISSUES WITH METHOD OF ANALYSIS

L. F. EBELL

Forest Research Laboratory, Canada Department of Forestry and Rural Development, Victoria, B.C.

(*Received* 11 *June* 1968)

Abstract—Techniques are described for sample preparation and analysis of total ethanol-soluble sugars of conifer tissue, using a stabilized, diluted anthrone reagent. This reagent gave similar values for the principal free sugars, in contrast to a greater divergence with other anthrone reagents and with the phenol–sulphuric method. Non-carbohydrate interfering substances caused serious overestimations of the sugar content of fully cleared samples by a reducing sugar method. Deproteinizing was not required for colorimetric methods but decolorizing with charcoal was essential, largely to remove interfering phenolic compounds.

INTRODUCTION

OUR STUDIES in the physiology of reproduction of Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) have included checking seasonal changes in carbohydrate food reserves in buds, foliage, twig-bark and wood accompanying formation of girdling-induced reproductive primordia. Because of the large number of samples involved, and the small sample size of some tissues, rapid semi-micro methods of analysis were necessary. Precision also was essential in order to reveal small differences between treated and control stems of double-trunked trees used as experimental material.

Experience has shown that conifer material presents unique problems in terms of physical characteristics and serious levels of non-carbohydrate interfering substances. Sample handling and the anthrone procedure adopted are described, and comparison is made with results obtained by other methods for total soluble sugar.

METHODS

Sample Preparation and Separation of Soluble Sugars From Polysaccharides

Samples are freeze-dried, weighed, ground in an intermediate Wiley mill to pass through a 40-mesh sieve, bottled and re-dried before analysis.

Duplicate 100 mg dry samples are weighed into thimbles folded from 9-cm Whatman GF/A glass-fibre filter papers, and extracted in microsoxhlet assemblies for 4 hr with 20 ml of 80% ethanol. Over 90 per cent of the soluble sugars are extracted in the first hour, and third-hour extracts give little or no color with anthrone reagent. Use of glass thimbles eliminates errors from cellulose fibre contaminants and minimizes atmospheric moisture problems associated with weighing paper thimbles. This simplifies weighing the extracted sample and the calculation of total ethanol-extractable substances.

A specially constructed rotary soxhlet assembly permitted extraction of samples in groups of twelve. This number, or multiples, are conveniently handled together throughout analysis

of total soluble sugars. The soxhlet thimbles containing the residue may be air-dried, and utilized for starch analysis.

Analysis of Total Soluble Sugars

Ethanol is evaporated from the extracts on a steam bath, with small additions of water near the completion of evaporation to maintain the extracted material in solution. The extract is quantitatively transferred to 50-ml centrifuge tubes and made up to 20 ml.

Extracts may be deproteinized with 1 ml of saturated, neutral lead acetate, and excess lead removed with 2 ml of saturated disodium phosphate. The adequacy of these volumes may be verified by centrifuging and checking for further precipitate on addition of drops of appropriate reagent. Insufficient de-leading leads to turbidity with the anthrone reagent and erratic results. A more essential clearing step, following the optional addition of deproteinizing agents, involves decolorizing with approximately 200 mg of powdered bone charcoal and vortex mixing tube contents several times over a 10-min period. The influence of clearing procedures will be discussed later. The extracts are now centrifuged and the supernatant filtered through a 1-in., 0·8 μ pore size cellulose triacetate membrane into 50–200-ml volumetric flasks placed in vacuum filtrators. Flask size is selected to bring final sugar concentrations within a preferred range of 30–100 μg per ml. The packed precipitate and charcoal retains almost 1·5 ml of water, in which 10 per cent of the total sugars could theoretically be dissolved. This is easily recovered by thoroughly re-suspending the precipitate with 15–20 ml of water, recentrifuging, and combining the supernatants. This single washing was found to reduce the sugars remaining with the precipitate to about the detectability level with anthrone. Attempts to wash the precipitate by filtering techniques alone proved time-consuming and yielded erratic results and recoveries of added glucose.

An anthrone procedure was selected for its simplicity, reproductibility and its direct applicability for estimation of total soluble sugar food reserves in large numbers of extracts containing high proportions of non-reducing sugars.

One ml of sample, standard or water blank, in 20 × 150 mm tubes, is reacted for exactly 7·5 min in an actively boiling water bath with 10 ml of 0·15% anthrone reagent (prepared by dissolving 1·5 g anthrone in 1 l. of a solvent mixture composed of 1 vol. 20% ethanol: 3 vol. concentrated, reagent grade concentrated H_2SO_4). The effect of time lag in treating a large test-tube rack of samples in a test series, and variations due to heat of dilution is eliminated chiefly by rapid addition and mixing of cold reagent with a repetitive type dispensing syringe, and because the acid content of the reagent is already diluted to 72 per cent of concentrated. Samples are chilled in an ice bath during reagent addition and after the timed heating period. The test series is then brought to room temperature and absorptivity determined at 625 nm. Concentration of sugar is calculated as glucose from a standard curve (prepared from 0 to 100 μg/ml D-glucose standards in saturated benzoic acid solution) which were determined daily.

Identical colorimeter readings were reliably obtained from aliquots of the same extract. Repeatability of the method is primarily limited by sample preparation steps. The standard deviation from duplicate extractions of 30 samples was ±0·10% sugar for twig axis samples and ±0·18% sugar for foliage samples, dry weight basis. This was equivalent to a coefficient of variation (95 per cent level of confidence) of ±3·7 and ±3·4 per cent, respectively, of the mean soluble sugar content of these tissues. In practice, results were accepted when duplicate analyses agreed to within 5 per cent of the smaller value.

Our tests of the numerous modifications of anthrone and sulphuric acid concentrations,

and conditions of color development that have been proposed, and their effect on reagent stability, reaction color intensity and stability, led to adoption of similar test conditions and reagent formulation as that used by Fales.[1] This diluted acid reagent produces a greater color intensity than the customary undiluted acid reagents, and increases reagent stability by reducing the rate of anthrone decomposition to the brownish cast of its parent substance, anthroquinone. Ethyl sulphate ester formed from the 5 per cent final content of ethanol in the Fales reagent also contributes greatly to reagent stability, possibly due to formation of a more stable equilibrium of anthrone with its chromogenic anthranol form. Ethanol proved more effective than thiourea, suggested by Roe,[2] as an anthrone reagent stabilizer.

The reagent, if refrigerated, can be stored in quantity and the same standard curve validly used for 2 weeks or more. The reagent remains light yellow in color and unused portions can usually be combined with the next batch. Anthrone itself was found to be subject to deterioration after lengthy shelf storage. Lots that displayed a green tinge on dissolving, indicating the presence of excessive amounts of the active anthranol tautomer, were avoided due to higher blank readings and reduced reagent stability.

Comparison of Anthrone Method with Other Methods for Total Soluble Sugars

Cleared and uncleared non-hydrolyzed extracts of Douglas fir twig-bark were analysed in triplicate by the preceding anthrone procedure and the ferricyanide oxidation–ceric sulphate titration method of Hassid.[3,4] Sugars were removed from 85-ml aliquots of these extracts by 48-hr fermentation with 4 ml of a 5% suspension of triple-washed yeast, and the analyses repeated to determine the degree of interference from non-carbohydrate substances, a procedure similar to that used by Heinze and Murneek.[5]

Deproteinizing steps are frequently omitted with anthrone procedures. However, the characterization of the tryptophane–carbohydrate–anthrone complex by Tuller and Keiding,[6] and Shetlar's[7] listing of other nitrogenous interfering substances made these steps seem essential, particularly for samples that are low in sugars. The data of Table 1 show that the anthrone method, as well as the reducing sugar method, gave erroneously high results when the extracts were not cleared. Turbidity of uncleared extracts was a significant error source.

TABLE 1. COMPARISON OF THE ANTHRONE COLORIMETRIC METHOD AND THE FERRICYANIDE OXIDATION–CERIC SULPHATE TITRATION REDUCING SUGAR METHOD ON NON-HYDROLYZED EXTRACTS OF DOUGLAS FIR TWIG BARK

	% Total reactants		% Reactants after 48 hr yeast fermentation		% Net sugars as glucose	
Treatment	Anthrone	Reducing sugar	Anthrone	Reducing sugar	Anthrone	Reducing sugar
Not cleared	8·2	18·6	1·0	5·8	7·2	12·9
Cleared	6·9	8·6	0·6	2·7	6·4	5·8

[1] F. W. FALES, *J. Biol. Chem.* **193,** 113 (1951).
[2] J. H. ROE, *J. Biol. Chem.* **212,** 335 (1955).
[3] W. Z. HASSID, *Ind. Engng Chem. Anal. Ed.* **8,** 138 (1936).
[4] W. Z. HASSID, *Ind. Engng Chem. Anal. Ed.* **9,** 228 (1937).
[5] P. H. HEINZE and A. E. MURNEEK, *Res. Bull. 314, Missouri Agr. Exp. Sta.* (1940).
[6] E. F. TULLER and N. R. KEIDING, *Anal. Chem.* **26,** 875 (1954).
[7] M. R. SHETLAR, *Anal. Chem.* **24,** 1884 (1952).

The 0·6% anthrone reactants in the cleared extracts after yeast fermentation may represent non-fermentable oligosaccharides[8] and, if so, are validly included in the total anthrone reactants. Non-reducing sugars that ferment slowly would not be included in the reducing sugar method results, and the 3% level of reducing sugar method reactants after yeast fermentation represents non-carbohydrate interfering substances. The net sugar values by both methods appear comparable from the cleared extracts, with the differences accountable by inclusion or non-inclusion of the non-reducing sugar components. If only total reactants before fermentation are considered, the ferricyanide oxidation method seriously overestimated the sugar content of cleared extracts of Douglas fir bark.

In addition to the necessary hydrolysis, more rigorous and complex clearing procedures, such as the mercury–zinc routine proposed by Friedemann *et al.*,[9] appear necessary before using reducing sugar methods for conifer twig tissues.

The phenol–sulphuric acid method of Dubois *et al.*[10] was tested for use with uncleared Douglas fir tissue, since Barnett and Tawab[11] were able to determine lactose in dairy products without interference from proteins and amino acids. However, for our tissues, preliminary work showed that deproteinizing and decolorizing steps were more essential using phenol–sulphuric and with *p*-aminobenzoic acid[12] procedures than for the anthrone method. The *p*-aminobenzoic method gave values on cleared twig-wood extracts which were only 55 per cent of anthrone values. Since much longer reaction times were involved, the method was not investigated further.

Duplicate 100-mg samples of new and 1-yr-old foliage and twig axis tissues of Douglas fir and grand fir (*Abies grandis* (Dougl.) Lindl.), sampled in mid-June, were extracted and made to 25 ml without clearing. Then 5-ml aliquots of each of the eight samples were left uncleared or treated at one-quarter the usual level with three clearing procedures. These were then made up to 25 ml for a final dilution factor of 125 and analysed with the anthrone and with the phenol–sulphuric procedures. As shown in Table 2, lead acetate–sodium diphosphate treatment alone gave soluble sugar values which were intermediate between uncleared and fully treated extracts. Values from charcoal alone and from lead plus charcoal treatment were statistically identical, showing that interfering substances were adequately removed by adsorption on charcoal. Lead acetate precipitation steps could be deleted with these specific tissues as constituting a needless increase in preparation time and an added source of handling error.

Comparisons between sample types showed inadequate clearing caused more serious overestimation of sugar values of Douglas fir than grand fir samples, of 1-yr-old tissue than new tissue, but caused similar overestimations for twig axis tissues as for foliage despite differences in pigmentation. The characteristics of these samples and the greater susceptibility of the phenol–sulphuric method to improper clearing suggest that native phenolics constitute a major class of interfering substances in colorimetric determination of sugars. Phenolics in the alcohol solubles of Douglas fir have been described by Barton.[13] Most interference may result from the color and turbidity of condensation products, but also from direct chemical reaction with reagents. Native phenols would augment the phenol levels in

[8] H. GIRARD and E. DE CHARON, *Ann. Inst. Natl. Rech. Agron.*, Ser. E., *Ann. Tech. Agric.* **8,** 55 (1959); from *Biol. Abst.* **36,** 21511 (1961).
[9] T. E. FRIEDEMANN, C. W. WEBER and N. F. WITT, *Anal. Biochem.* **6,** 504 (1963).
[10] M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS and F. SMITH, *Anal. Chem.* **28,** 350 (1956).
[11] A. J. G. BARNETT and G. A. TAWAB, *J. Sci. Food Agric.* **8,** 437 (1957).
[12] B. LEOPOLD, *Anal. Chem.* **34,** 170 (1962).
[13] G. M. BARTON, *Can. J. Botany* **45,** 1545 (1967).

the phenol–sulphuric method, the color produced by phenols and sulphuric acid contributes to absorption levels at the wavelength used for the anthrone method, and Dubois *et al.*[10] have noted that phenol interferes with formation of the green color of the anthrone reaction.

Phenolic substances vary as to whether they are precipitated by lead acetate. Evidently charcoal treatment more specifically removed those substances causing error with present samples and procedures. In work to be reported separately, charcoal was found to be the most efficient means of removing phenolic substances inhibiting enzymes used for starch glucose determination.

The numerous soluble sugars can be considered as being largely interconvertible as energy sources or metabolites within the living plant. The ideal total sugar method should measure

TABLE 2. INFLUENCE OF CLEARING TREATMENTS ON PER CENT SOLUBLE SUGAR VALUES OF CONIFER TISSUES BY TWO COLORIMETRIC METHODS

	Anthrone				Phenol-sulphuric			
	Not cleared	Lead acetate	Charcoal	Lead and charcoal	Not cleared	Lead acetate	Charcoal	Lead and charcoal
Douglas fir sampled June 18								
New foliage	9·4	8·9	7·4	7·4	10·9	9·7	6·8	7·1
1-yr foliage	7·9	7·2	6·1	5·6	10·1	8·7	5·8	5·0
New twig bark and wood	8·2	7·9	7·2	7·3	10·5	10·0	6·2	6·6
1-yr twig bark	6·4	5·6	4·7	4·5	7·8	7·4	4·6	4·0
Grand fir sampled June 13								
New foliage	6·9	6·1	4·8	5·1	7·3	6·5	4·1	4·5
1-yr foliage	9·0	8·3	7·8	7·8	10·2	8·4	6·9	7·0
New twig bark and wood	5·9	5·3	5·2	5·2	7·6	6·2	4·6	4·9
1-yr twig bark and wood	4·7	4·0	3·8	3·9	6·3	5·1	3·8	3·8
Mean of all tissues	7·3	6·7	5·9	5·8	8·8	7·8	5·4	5·4

each sugar equally, in contrast to the very different reaction rates and relative reducing capacities or formation of furfural derivatives on which most available techniques are based. This difference in reactivity, expressed as glucose equivalents per unit weight, is shown in Table 3. The most similar reactivity over a range of sugar types is obtained by reducing sugar methods, due to the stability of the oxidized sugar after an adequate time of heating.[5, 14]

In contrast, fading in the heating bath after the very different optimum heating periods for each sugar is a characteristic of anthrone procedures.[15, 16] With the present procedure, fading of the faster reacting fructose and sucrose occurred to a degree where their color intensities coincided with that of glucose at 7·5 min of heating. Shorter or longer heating

[14] T. E. FRIEDEMANN, C. W. WEBER and N. F. WITT, *Anal. Biochem.* **4**, 358 (1962).
[15] L. H. KOEHLER, *Anal. Chem.* **24**, 1576 (1952).
[16] T. NARASAKI, *Tech. Bull. Fac. Agr. Kagawa University, Japan* **11**, 265 (1959).

periods increased differences between these three main sugars of Douglas fir extracts. Raffinose is slightly underestimated due to the low reactivity of its constituent galactose, and xylose is seriously underestimated due to rapid fading of pentose–anthrone color after very short optimum reaction periods. As these sugars were never present in large quantity, this underestimation did not seriously affect total sugar results. The stronger anthrone reagent used by Koehler[15] as a means of distinguishing between sugars resulted in a greater reported divergence. The phenol–sulphuric acid method of Dubois *et al.*[10] gave poorer correspondence between sugars than the anthrone methods. The behaviour of pentose sugars with the phenol–sulphuric acid method parallels the greater absorbance with pentoses, compared to hexoses, obtained with short heating with anthrone, suggesting that reaction rate and fading of various sugars in both methods may involve similar mechanisms. This further suggests that the phenol–sulphuric acid method could also be improved by use of a more dilute acid and by substituting controlled heating for the heat of mixing technique presently employed.

TABLE 3. VARIATION IN GLUCOSE EQUIVALENTS, PER UNIT WEIGHT, OF VARIOUS SUGARS WITH DIFFERENT METHODS OF ANALYSIS

	Present author		Koehler[15]* 0·2% anthrone in conc. H_2SO_4		Friedemann *et. al.*[14] reducing sugar methods	
Sugar	Phenol–sulphuric heat of mixing	0·15% anthrone in 72% H_2SO_4, 7·5 min heating	7·5 min heating	12 min heating	Ferricyanide	Cupric tartrate
D-Glucose	100	100	100	100	100	100
D-Fructose	82	103	128	100	99	95
Sucrose	94	104	128	128	—	—
Raffinose	85	91	—	—	—	—
D-Galactose	60	51	71	69	78	80
D-Xylose	125	25	4	4	97	96
D-Ribose	120	12	3	3	86	77

* Approximate values from graphs.

EXPERIMENTAL

Freeze-Drying of Conifer Tissue

Bell[17] has reviewed the precautions necessary in sample preparation for carbohydrate analysis, including the problem of transient stimulation of enzymes during the warming-up period of oven drying and the potential heat destruction of carbohydrates. For these reasons a sample handling system has been adopted based on rapid field dissection, transportation in a dry-ice refrigerated field chest and fragmentation of frozen foliage to 5 mm or smaller pieces with a sharpened stainless-steel pipe, followed by freeze-drying. The latter is conducted in a high capacity chamber fitted with quick-disconnect heat exchanger shelves. Shelf heat is supplied to maintain sample temperature between $-5°$–$-10°$ during the early period of rapid water loss and high rate of evaporative cooling; it is then adjusted to room temperature. More than 90 per cent of the total water content of Douglas fir foliage is removed before thawing takes place, and desiccation is 99 per cent complete by the time samples warm to room temperature. Shelf heat is then raised to 35° for a 24-hr secondary drying period, analogous to vacuum oven drying.

Slightly more water and other volatiles are removed from Douglas fir foliage with freeze-drying than with 70° oven drying. Wood, bark and root tissues freeze-dry rapidly, but foliage may require up to 60 hr total drying time, with considerable variation between trees attributable to large differences in amounts of resinous

[17] J. D. BELL, in *Modern Methods of Plant Analysis* (edited by K. PAECH and M. V. TRACEY), Vol. 2, p. 1, Springer-Verlag, Berlin (1955).

inclusions which tend to clog water vapor migration pathways. Buds freeze-dry well, but have to be ground subsequently at low temperatures due to high levels of screen-clogging resins which do not harden as with heat drying. After grinding, samples are again freeze-dried to remove atmospheric moisture picked up during weighing, grinding and other handling. This re-drying follows a similar pattern to the latter stages of the initial drying, with the added precaution that chamber evacuation rate must be cautiously regulated to permit time for de-gassing and avoid blowing of the powdered samples.

The extra time involved in freeze-drying, as compared to the simplicity of oven drying, is partially compensated for by an added degree of confidence in the samples, which have superior color and physical characteristics. In subsequent handling it must be remembered that enzymes are not denatured by freeze-drying and can affect later results if the sample is directly exposed to water.