Carbohydrate Research, 191 (1989) 175–189
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

QUANTITATION OF FREE SUGARS IN PLANT TISSUE BY G.L.C. OF THEIR PERACETYLATED ALDONONITRILE AND KETOXIME DERIVATIVES

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(Received October 22nd, 1987; accepted for publication, in revised form, April 17th, 1989)

ABSTRACT

The assay of free sugars and related compounds in plant tissues has been studied by use of a defined procedure involving cold ethanol extraction and conversion of the extracted saccharides to the peracetylated derivatives of aldononitriles, ketoximes, alditols, and nonreducing sugars. Quantitation of the resulting mixture was accomplished by g.l.c. separation and computer-performed integration, referenced to an internal standard. Linearity of detector response, and the coefficients of variation of the detector response, were examined for a representative aldose (D-glucose), ketose (D-frutose), nonreducing sugar (sucrose), and alditol (D-glucitol). Retention times and detector responses for 39 additional saccharides and alditols were measured under identical conditions.

INTRODUCTION

Described herein is a general method for establishing the percent wet-weight of free sugars and related compounds in plant tissues. This method incorporates sugar extraction, derivatization, chromatographic separation, and digital integration referenced to an internal standard. The derivatization procedure converts aldoses into peracetylated aldononitriles (PAAN), ketoses into peracetylated ketoximes (PAKO), and peracetylates the nonreducing saccharides. A major advantage of the PAAN and PAKO derivatives lies in the extensive structural data which can be obtained from mass spectral studies on the chromatographic peaks of unknown saccharides[1,2]. Therefore, the objective herein is to describe a simple analytical approach to the survey and quantitation of the saccharides present in extracts of plant tissue, with provision for the identification of unknown compounds indicated by extra chromatographic peaks.

Lance and Jones[3] first used chromatographic separation of PAAN derivatives for the study of partially *O*-methylated D-xyloses from the hydrolysates of D-xylans. Dmitriev *et al.*[4] both separated the PAAN derivatives of nonmethylated saccharides, and investigated the electron-impact mass spectrometry (e.i.-m.s.),

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0008-6215/88/$ 03.50

but not the chromatography, of partially *O*-methylated derivatives of D-glucose. Varma and coworkers[5] extensively used PAAN derivatives to survey the saccharide content of hydrolysates from glycoproteins of mammalian origin. Szafranek *et al.*[6,7] studied the e.i.-m.s. fragmentation pathways of PAAN derivatives, and also demonstrated that these derivatives could be simultaneously chromatographed, at good resolution, with alditol acetate derivatives of saccharides by the use of capillary columns. Baird *et al.*[8] have used the simultaneous g.l.c. separations of PAAN and alditol acetate derivatives for the analysis of products from Smith degradations. Belcher *et al.*[9] analyzed the total sugar content of the gossypol glands in cotton anthers (after hydrolysis) by the PAAN procedure, and Nicollier *et al.*[10,11] measured the component sugars (glucose and rhamnose) in two new flavonoids extracted from the flowers of white clover (*Melilotus alba*). Schultz *et al.*[12] monitored the formation of sugars in the enzymatic hydrolysis of the hemicelluloses from the steam explosion of a variety of materials (hard wood, rice hulls, etc.). Hedin *et al.*[13] compared the chemical composition of whorl tissue from resistant and non-resistant strains of corn (*Zea mays* L.) in studies on susceptibility to the southwestern corn borer. In a similar manner, Maningat and Juliano[14] identified the component sugars in the cell walls of rice bran. Both Whitfield *et al.*[15] and Pringle *et al.*[16] analyzed the composition of exopolysaccharides and lipopolysaccharides from microbial strains (respectively, *Xanthomonas campestris* and *Pseudomonas fluorescens*). Gage *et al.*[17] used a combination of ^{31}P-n.m.r. and the PAAN procedure to identify a phosphomannan in the cell wall of the yeast *Saccharomyces cerevisiae*. The PAAN procedure has also been applied to sugars which arise under conditions that are profoundly different from those involved in the extraction of natural products, and which are contaminated with impurities. Nyhammar *et al.*[18] used PAAN derivatives to study the products which arose through the participation of the Strecker degradation in the Maillard reaction. Shafizadeh and co-workers routinely employed the procedure to examine products arising from the pyrolysis of polysaccharides[19] and wood[20,21]. In these studies, sugars arose from the hydrolysates of larger molecules which had been separated from plant tissues. Free sugars, that is components extracted from plant tissue without the breaking of covalent bonds, were not analyzed. Maningat and Juliano[14] extensively used the PAAN procedure for the analysis of sugars from hydrolysates, but used wet chemistry to analyze for the free-sugar content of the tissues.

In addition to the above-mentioned conventional analyses, there are several reports of the use of the PAAN procedure with g.l.c.-m.s. to identify new saccharides occurring in natural polymers. Jackson *et al.*[22,23] found 3-*O*-methyl-L-rhamnose in hydrolysates of extracellular and capsular polymers from strains of *Rhizobium*. Similarly, Mort and Bauer[24,25] identified 4-*O*-methylgalactose in extracellular and capsular polysaccharides of *Rhizobium japonicum*. Romanowska *et al.*[26] characterized 4-deoxy-D-*arabino*-hexose as a component of a lipopolysaccharide isolated from a strain of *Citrobacter*, and Edwards *et al.*[27] identified intracellular mannitol as a product of the metabolism of D-glucose in *Staphylococcus aureus*.

The PAAN procedure has been extensively used for the final part of methylation analyses, in which the derivatives separated are *O*-methylated, *O*-acetylated aldononitriles. Recent, demanding structural analyses of heteropolysaccharides using these derivatives include: carrageenan (D-galactose, 3,6-anhydro-D-galactose) by Matulewicz and Cerezo[28], xanthan gum (D-mannose and D-glucuronic acid) by Cadmus *et al.*[29], and an arabinogalactan from *Larix sibirica* by Karásconyi *et al.*[30].

A vigorous development and application of the PAAN procedure is indicated by the foregoing survey of the literature, in which several features are evident. Firstly, there are few applications of the PAKO method to the analysis of ketoses, or of the PAAN method to disaccharides. Secondly, the PAAN–PAKO procedure has not been used for the direct analysis of free sugars from plant tissue. The literature also shows that studies on plant tissue often do not include estimates of the free sugars present, and when these are determined wet chemistry is often used, with saccharides being reported in terms of "total reducing" and "total nonreducing" sugars. However, extensive studies have been done on the free-sugar content of commercial fruits, and these data have been summarized by Wrolstad and Shallenberger[31]. It has been concluded that the free sugars in the tissues of plants are mainly limited to D-glucose, D-fructose, and sucrose, with sucrose playing the major role in the transport of energy[32]. A derivative of D-glucose, the alditol D-glucitol, has also been described as involved in energy transport in plants[33]. In addition to D-glucitol and related alditols, other low molecular weight carbohydrates in plant tissues include the cyclitols and various glycosides.

Therefore, the objectives of this work were twofold. The first was to establish an analytical method that will quantitate the major free saccharides of plants (D-glucose, D-glucitol, D-fructose, and sucrose). The second was to retain the features of the PAAN–PAKO procedure which both identify unusual or unexpected sugars and allow sophisticated mass-spectrometric structural determinations of these compounds[1,2].

RESULTS AN DISCUSSION

The data presented here are summaries of computer calculations from the Varian 401 data processing system, based upon chromatograms similar to the example of Fig. 1. In all studies, chromatography was performed with a chloroform solution of the derivatives of L-rhamnose, D-glucose, D-glucitol, D-fructose, and sucrose. Table I summarizes a typical set of data produced by the repetitive injection of 1 μL aliquots of the chloroform solution, and by uniformly employing the chromatographic and integration methods described below. No "percent wet-weight" could be calculated, as the chloroform injection solution resulted from the derivatization of known weights of pure sugars dissolved in pyridine. Appropriate parameters were programmed into the computer, and all chromatographic and

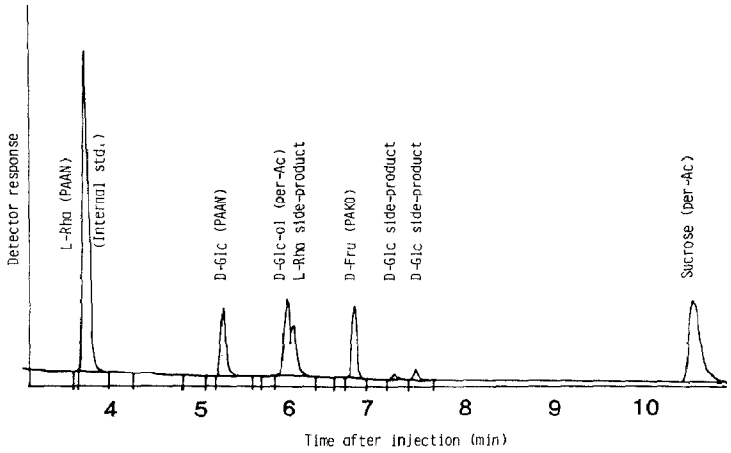

Fig. 1. Chromatogram of a mixture containing the PAAN derivative of L-rhamnose (reference) and the derivatives of the four major saccharides studied by the PANOS-GR method.

computational operations of a typical analytical procedure were performed for each run. The areas of four chromatographic peaks, representing the major derivatives from D-glucose, D-glucitol, D-fructose, and sucrose, were electronically compared to the area of the derivative of L-rhamnose, which was defined as the internal standard. The internal standard method requires a chromatogram of a reference solution for calibration of the detector response, and for this purpose a chromatogram resulting from the foregoing mixture was used. All peaks in the chromatograms summarized in Table I approximated the area count expected from a plant tissue containing one percent wet-weight of each of the four saccharides. The peak representing the derivative of L-rhamnose had approximately the same area as the other four major peaks, producing a count of 1.8 million arbitrary integration units, and (for 1 μL) corresponding to an injection of 0.8 μg of underivatized L-rhamnose. Side products were present in the chromatogram, from L-rhamnose and D-glucose (see Fig. 1), but these did not interfere with the quantitation process. A

TABLE I

RESULTS FROM NINE REPLICATE INJECTIONS OF DERIVATIVES OF THE SAME REFERENCE SOLUTION

Parameter	*Hypothetical percent wet weight*[a]			
	D-*Glucose*	D-*Glucitol*	D-*Fructose*	*Sucrose*
Mean	105.4	72.8	115.4	85.8
Range	3.0	2.2	2.8	1.3
Std. dev.	1.1	0.8	2.8	1.3
C.v.[b]	1.1	1.1	2.4	1.5

[a]The hypothetical percentages were normalized at approximately 100% for each saccharide. [b]Coefficient of variation.

TABLE II

RESULTS FROM TRIPLICATE INJECTIONS OF THE DERIVATIVES FROM FIVE PARALLEL TREATMENTS OF THE SAME REFERENCE SOLUTION

Parameter	*Hypothetical percent wet weight*[a]			
	D-*Glucose*	D-*Glucitol*	D-*Fructose*	*Sucrose*
Mean	108.7	74.5	117.1	84.7
Range	5.7	5.0	10.0	1.7
Std. dev.	1.0	2.0	4.0	1.4
C.v.[b]	0.9	2.7	3.4	1.6

[a]The hypothetical percentages were normalized at approximately 100% for each saccharide with parameters identical to those used for the data in Table I. [b]Coefficient of variation.

coefficient of variation (c.v.) was established for each derivative, and found to be in the range 1 to 3 for this series of determinations (Table I). These values indicate the degree of reproducibility of the analyses when only the chromatographic and the computational system are involved.

To simplify the discussion in this and later reports, acronyms are used for the three major experimental steps employed. The plant-tissue extraction procedure described in the Experimental section, which uses shear and sonication, is defined as the SS method. The two-step derivatization procedure (including the chloroform extraction) is defined as the PANOS method — as a group of peracetylated aldononitriles, ketoximes, and (nonreducing) sugars can be produced. The final step, involving g.l.c. and a recording integrator, is described as the GR method. The combined procedure is described as the SS-PANOS-GR method. In Table I the data were generated by a combination of the PANOS and GR methods.

Table II summarizes data from five independent derivatizations of aliquots of a pyridine solution containing known amounts of L-rhamnose and the four sugars under consideration. The solutions of sugars were derivatized in parallel, starting at reaction step 1, and the chloroform extracts were chromatographed and computed by the method just described. The extract from each derivative was chromatographed in triplicate, the results were averaged, and the data sets compared for agreement. Table II shows c.v. values in the range of 2 to 3, indicating that a modest degree of variation was introduced by the derivatization procedure (compare with the c.v.'s in Table I).

Table III summarizes data from four separate derivatizations of aliquots of a 90%-ethanol extract of a plant tissue — petals from the hawthorne (*Crategus* sp). This extract was employed because all four saccharides of interest are present at near 1% wet weight — a rare combination. All peaks observed in the chromatograms of the extract were attributable to one of the four major sugars. The determinations employed the same derivatization and chromatographic methods used to generate the data in Table II, but included the evaporation procedure (70°, <3 min) described in the Experimental section. The coefficient of variability calcula-

TABLE III

RESULTS FROM DUPICATE INJECTIONS OF THE DERIVATIVES FROM FOUR PARALLEL DERIVATIZATIONS OF AN EXTRACT OF THE PETALS OF HAWTHORNE (*Crategus* sp.)

Parameter	*Percent wet weight*			
	D-*Glucose*	D-*Glucitol*	D-*Fructose*	*Sucrose*
Mean	0.713	0.954	1.19	0.166
Range	0.020	0.011	0.040	0.007
Std. dev.	0.0089	0.0048	0.0018	0.0032
C.v.[a]	1.3	0.5	1.5	2.0

[a]Coefficient of variation.

tions indicate that error, beyond that associated with the derivatization procedure, was not introduced at this step.

The linearity of the detector response was evaluated for each of the four saccharides under consideration. A summary of the data is shown in Fig. 2. Two chloroform extracts, resulting from two independent derivatizations of the sugars dissolved in pyridine, were employed. The first chloroform solution resulted from the derivatization of a known amount of L-rhamnose, the second from the derivatization of known amounts of D-glucose, D-glucitol, D-fructose, and sucrose,

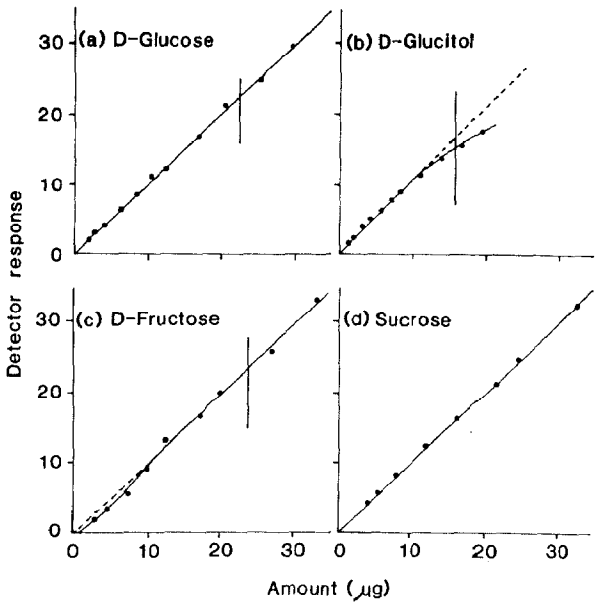

Fig. 2. The FID detector response of the Varian Vista system *vs.* the micrograms of injected sample (pre-derivatization weights) for each of the major sugars studied. The ordinates are the normalized area counts, in millions. The vertical bar in the above plots represents limit of the injection weight at which the analog-digital converter went over range, with loss of some area at the top of the peak.

respectively. These chloroform solutions were combined, using Langlevy pipettes, in dilution ratios of 0.25 to 3.5 — allowing a linearity study over 1.4 orders of magnitude. The volumes of the chloroform solution injected varied, so that the same amount of L-rhamnose derivative (corresponding to 0.8 μg of starting sugar) was injected each time. The detector responses, as described below, are based upon the relationship of the *weight of the starting, underivatized saccharide* to the area count under the chromatographic peak, normalized to that for L-rhamnose by the defined integration program.

Regression analyses were performed upon the data resulting from the injection of varying amounts of sample in the proposed region of linearity for each of the four saccharides under consideration. All the following data are based upon two injections of each solution, and the averaging of the two computed responses.

Fig. 2a shows linearity for D-glucose in the 2 μg through 30 μg region. Regression analysis of eleven uniformly spaced values (for amount of sugar) in this region gave an R-square value of 0.9976, and a corresponding c.v. of 3.8. (When amounts in the 2 μg through 12 μg region were used, a c.v. of 2.2 resulted.) For D-glucitol, Fig. 2b shows linearity in the 1 μg through 10 μg region. Regression analysis for seven uniformly spaced amounts of D-glucitol in this region indicated an R-square value of 0.9867, and a corresponding c.v. of 7.4. (When the amounts of D-glucitol in the 1 μg through 6 μg region were used, a c.v. of 4.3 resulted.) The detector response for D-glucitol rapidly deviates from linearity in the 10 μg to 20 μg region, and uncorrected measurements in this region could result in severe underquantitation. On the other hand, data plotted in Fig. 2c show that D-fructose deviates from linearity below the 8 μg level, and uncorrected measurements in this region will also be underquantitated. Regression analysis of eight uniformly spaced values in the 9 μg through 42 μg region for D-fructose indicated an R-square value of 0.9885, and a corresponding c.v. value of 5.6. Finally, Fig. 2d shows a region of linearity for sucrose in the 4 μg to 60 μg region. Regression analysis for eleven uniformly spaced amounts of sucrose indicated an R-square value of 0.9994, and a corresponding c.v. of 1.9. Though the c.v. values from linear regression are not mathematically identical to the c.v. values from a single data set (*e.g.*, data for a specific sugar in Table I), a similar effect at a similar level of confidence is being measured.

The foregoing data indicate that the quantitation of mixtures of D-glucose, D-glucitol, D-fructose, and sucrose can be achieved with a coefficient of variation under 3%, and in many cases under 2%. Importantly, two critical measurements were always taken for each datum in the above tables — the area of the peak for the internal standard, and the area of the peak for the sugar under study. For measurements in Tables I–III, the amount of injected internal standard was approximately 0.8 μg (based upon starting L-rhamnose) and was held constant, by use of a Chaney adaptor, to within a few percent for each injection. Independent studies (data not shown), with the amount of D-glucose held constant and that of L-rhamnose varied, indicated that the response of L-rhamnose in the 0.2 μg to 10

μg region had the same degree of linearity as that of D-glucose. The c.v. studies were a major reason for maintaining a uniform size for the peak area of the internal standard — as the measurement of the c.v. for each unknown is dependent upon the area measurement of the reference peak. The amount of standard injected was held close to that approximating 1.0% wet weight of L-rhamnose in an extract of plant material. The determination of any unknown is dependent upon the c.v. of the reference peak, in this case about 0.02 for L-rhamnose. For example, Table III summarizes the quantitation of D-glucitol at about 1.0% wet weight; in this case the D-glucitol peak is related to an internal standard peak of similar area. The sucrose content of these samples is in the 0.2% range, but sucrose is still referenced to the same standard peak. Therefore the data are again expected to have a c.v. of 0.02 (based upon the 1% wet-weight value of the reference standard) and the expected c.v. of this sucrose determination is still 0.02 of 1.00 wet-weight percent.

The data from Tables I–III and Fig. 2 combine to indicate that standard deviations in the range of 0.02% wet weight can be expected if the following approach is taken: (a) use of the PANOS-GR procedure, with g.l.c. peaks within the range of linearity described for each compound; (b) adjustment of the internal standard peak to correspond to 0.8 μg of L-rhamnose; and (c), averaging the results of duplicate injections of the derivatized sample. By varying the ratio of the volume of the internal standard (pyridine) solution to the volume of the aliquot of the tissue extract the regions of linearity in Fig. 2 can correspond to any actual percent wet weight of saccharide. In a similar manner, control over sample size is achieved by modifying the ratio of tissue weight to the final volume of 90%-ethanol extract.

Two important areas are not discussed here. The first is the efficiency of the extraction procedure (employing high shear and ultrasonication) with actual plant tissues. The second is the compatibility of the derivatization procedure with extracts from a wide range of plant material. These questions are being examined, and the current status of the research can be summarized as follows. Tissue from approximately 200 species of herbaceous plants and woody plants (both deciduous and coniferous) have been studied without interference. The range of variability of saccharide content in tissues of plants growing under natural conditions is as large as, or greater than, the c.v. levels established by this procedure.

Several parameters of the SS-PANOS-GR procedure are not critical. Precise reaction times are not important — 5 minutes less and 15 minutes more have little effect on quantitation. Change in the chloroform:water ratio has little effect when an internal standard is used. The chloroform extracts are stable for longer than a week. Once calibrated with a known reference solution, the g.l.c. columns retain detector responses which change little on a day-to-day basis. Data with similar c.v. agreement have been taken on different, but similarly packed, columns. The columns have an extended life; the data reported here were taken with a column which had previously been used for several hundred similar chromatographic determinations.

Other parameters are critical for the quantitative application of the method

to plant tissue. The specific OV-17 packing (defined for the EPA 625 procedure) is important — several alternative OV-17 packings either cause serious peak tailing, or result in serious underquantitation of derivatives of the ketoses. The total amount of packing (column length and diameter) is important, as the regions of detector linearity have been defined in these terms. The pyridine:acetic acid ratio of the solvent is important. Derivatization of plant extracts requires the volume of acetic anhydride to be greater than that of the pyridine during the second reaction step — otherwise, many plant extracts yield chromatograms without peaks for aldoses and ketoses. Before the chloroform–water extraction procedure, the pyridine:acetic acid ratio must be adjusted to an excess of pyridine. Excess acetic anhydride during the extraction both greatly reduces the stability of the products in the final chloroform solution, and also shortens the life of the g.l.c. column packing (in terms of lowered detector responses for the PAKO derivatives). The stability of derivatives is increased by removing obvious water droplets from the final chloroform extract. The approach to digital integration is important. Lowering the rate of temperature increase, from 20°/min to 10°/min (requiring 20 min for a chromatogram), results in a sharp increase in the c.v. of data of the type shown in Table I. In our hands, the Varian 401 system produced data with smaller c.v. values than did several alternative systems. However, an older Varian 111 system produced data of a similar c.v. level. The rate of data sampling and the nature of the algorithms are apparently important for reproducible quantitation.

Reproducibility of quantitation can be achieved at, or near, the c.v. of the data in Table I without an internal standard if the following precautions are observed: (a) the volumes of water and chloroform are dispensed with 1% reproducibility for the extraction procedure; (b) no chloroform condensation step is used; and (c) injections are made with a Chaney adapter.

Table IV summarizes the retention times and relative response factors for a variety of derivatives of aldoses, ketoses, alditols, glycosides, and disaccharides — typical compounds anticipated in extracts from plant tissue. The identities of the peaks representing the saccharides and disaccharides were confirmed by chemical-ionization mass spectrometry, and were either the PAAN–PAKO derivatives (for reducing sugars), or the peracetylated derivatives (for nonreducing sugars). All values in Table IV are averages of at least two independent determinations, and the average c.v. of all replicate sets was 0.02. Though linearity studies were not done on these derivatives, the peak areas in all studies were within 20% of the L-rhamnose peak area at a 10 μg injection level, a level within the region of linearity for the four major saccharides studied. The response factor is the inverse of the peak area contributed by a given weight of a compound. Compared to the aldose reference (L-rhamnose), a given weight of alditol (*e.g.* D-glucitol) produces a g.l.c. peak approximately 50% larger, and a given weight of ketose (*e.g.* D-fructose) produces a g.l.c. peak about 30% smaller — possibly indicating that only a (reproducible) fraction of the starting aldoses and ketoses reaches the detector. All aldoses produced either one or two additional peaks (approximately 15% of the

TABLE IV

G.L.C. RETENTION-TIMES AND RELATIVE RESPONSE FACTORS FOR DERIVATIVES OF COMPOUNDS ANALYZED BY THE PANOS-GR METHOD

Parent compound	*Derivative type*[a]	*Retention time (min)*	*Response factor*[b]
D-Digitoxose	N	3.23	1.10
2-Deoxy-D-*erythro*-pentose	N	3.39	1.13
Methyl α-D-xylopyranoside	S	3.55	1.14
L-Rhamnose	N	3.80	**1.00**
6-Deoxy-D-glucose	N	3.93	1.41
L-Fucose	N	4.00	0.88
D-Ribose	N	3.88	0.96
D-Lyxose	N	3.96	0.94
D-Arabinose	N	4.03	1.12
D-Xylose	N	4.13	1.01
Ribitol	S	4.69	0.62
Arabinitol	S	4.78	0.67
Xylitol	S	4.91	0.70
2-Deoxy-D-*lyxo*-hexose	N	4.84	1.23
2-Deoxy-D-*arabino*-hexose	N	4.90	1.02
Methyl α-D-glucopyranoside	S	5.17	0.83
D-Allose	N	5.09	1.03
D-Talose	N	5.18	1.01
D-Altrose	N	5.24	1.43
D-Glucose	N	5.30	0.96
D-Mannose	N	5.25	0.97
D-Galactose	N	5.44	1.16
myo-Inositol	S	5.86	0.92
D-Mannitol	S	5.97	0.73
D-Glucitol	S	6.02	0.54
D-Galactitol	S	6.10	0.61
2-Acetamido-2-deoxy-D-glucose	N	6.17	1.20
2-Acetamido-2-deoxy-D-mannose	N	6.49	1.68
2-Acetamido-2-deoxy-D-galactose	N	6.51	1.68
D-*glycero*-D-*manno*-Heptose	N	6.41	1.02
D-Psicose	O	6.82	1.22
D-Fructose	O	6.83	1.15
D-Tagatose	O	6.90	1.38
L-Sorbose	O	6.92	1.36
D-*glycero*-D-*manno*-Heptitol	S	7.13	0.78
D-*manno*-Heptulose	O	7.83	2.24
Salicin	S	9.08	0.69
Maltose	N	10.30	1.37
Laminarabiose	N	10.40	1.45
Cellobiose	N	10.45	1.76
Sucrose	S	10.60	0.84
α,α-Trehalose	S	10.70	0.86
Gentiobiose	N	11.40	1.56

[a]N denotes a peracetylated aldonitrile, O a peracetylated ketoxime, and S a peracetylated nonreducing saccharide. [b]Value for the PAAN derivative of L-rhamnose taken as unity.

area) with retention times greater than the main peak. The area ratios and identities of these peaks are under study. No other saccharide, including the ketoses, in this study yielded more than one chromatographic peak. The extra peaks can be eliminated from the chromatograms by derivatization in anhydrous pyridine in the presence of zinc chloride. The additional peaks have not interfered with saccharide surveys, given their unique retention times and the resolution of the chromatography. The detector responses in Table IV are representative values established on a single column. Alternative columns gave similar values, but any column could show differences up to 5% from day to day. Thus, daily calibration is needed to achieve a precision of 0.02% wet weight. Though the columns are stable for hundreds of injections, a few injections of samples containing appreciable amounts of acetic anhydride will result in a column with very low detector responses for derivatives of either ketoses or hexosamines.

The above studies were done within the context of two additional areas of investigation on the PAAN–PAKO derivatization procedure. Firstly, both steps in the derivatization process are catalyzed by pyridine, or by pyridine-like compounds. PAAN–PAKO reaction conditions have been reported which employ *N*-methylimidazole[34] and 4-dimethylaminopyridine[35]. We have compared the PANOS procedure described here with methods employing catalysts for PAAN–PAKO derivatization, and without giving details may summarize the results as follows. We find that such catalysts provide little change in the speed of the reaction compared to pure pyridine, and that the shortened reaction times normally used with these catalyzed reactions can cause severe underquantitation of ketoses. Secondly, an important study by Furneaux[36] demonstrated that the PAAN derivatization was not quantitative, and drew attention to side products. However, the use of zinc chloride to eliminate the side products (see above) introduces uncertainty with regard to the reproducibility of reaction conditions, and is thus not recommended. On the positive side, the well-defined retention times and well-defined detector responses (relative to the detector response of the main peak) of the peaks from the side products can provide rapid confirmation of the identity of a given major peak (*e.g.* D-glucose).

In summary, a method has been described which: (a) allows quantitation of the saccharides present in plant tissues near the 1% level, (b) is rapid, allowing a survey from tetroses through disaccharides within a 15 minute injection to injection time, (c) is compatible with other components of plant tissue extracts, and (d) is very effective in quantitating ketoses. The derivatives retain the high asymmetry of the original saccharides and have small protective groups, making them useful for spectrometric analysis. The packed column system provides throughput of relatively large amounts of material, permitting studies of the effluent with detectors of low sensitivity.

EXPERIMENTAL

Sample extraction. — Plant tissue (1.00 g* ±0.01 g) was weighed into a 25 × 150 mm test tube. Iced 90% ethanol (6 mL) was added, and the tissue–ethanol mixture was subjected to (a) high-shear disruption (90 sec at 18,000 r.p.m.), the probe being washed with iced 90% ethanol (2 mL) and the solutions combined, and (b) ultrasonic disruption (30 sec at 35% of full power). The resulting suspension was transferred, with a 1 mL ethanol wash, into a 16 × 125 mm culture tube and centrifuged (3 min at 3,400 r.p.m.), and the supernate was decanted into a 10 mL volumetric flask. The residue was again extracted with 1 mL of 90% ethanol, and the supernates were combined and diluted to volume with 90% ethanol. A Janke and Kunkel Ultra-Turax 1P18/10S1 (also sold as a Tekmar STD Tissumizer), fitted with a 10 mm probe, was used for the shear-disruption step; a Fisher Sonic Dismembrator, Model 300 with a 4 mm probe, for the sonication; and a clinical centrifuge (International, Model CL) for the centrifugation.

Extract preparation. — An aliquot (0.50 mL*) of the ethanol extract was transferred to a 13 × 100 mm culture tube equipped with a 2 × 7 mm stir bar, and closed with a stopper carrying a 23 gage, 7.5 cm hypodermic needle as an air inlet, and a 2 mm (i.d.) glass tube attached to a high-volume vacuum pump. The culture tube was placed in a 70° heating block (Pierce Reacti-Therm heating-stirring module) and vacuum conditions were maintained which accomplished the evaporation of the solvent within 3 minutes. The glassware for volumetric transfers, both for extract preparation and for derivatization, was soaked for 30 min before use in a soap solution containing 1% ammonia.

Derivatization. Step 1. — (This procedure, and the following extraction, is conveniently done with sets of six samples). A "reaction solvent" was produced by weighing 166 mg* of L-rhamnose monohydrate and 7.54 mg of hydroxylamine hydrochloride into a 50 mL volumetric flask and diluting to volume with pyridine. The "reaction solvent" [200 μL* containing 598 μg of L-rhamnose (anhydrous basis) and 14.3 mg of hydroxylamine base] was added to the concentrated extract, and the culture tube was sealed with a Teflon-face screw cap, then placed in a heater-stirrer module at 69 ±1° for 20 min. After 2 min, solvent was swirled over the inner walls of the tube. *Step 2.* Acetic anhydride (0.25 mL) was added to the reaction mixture, and the sealed tube was again heated for 30 min at 69°. After the heating, pyridine (0.1 mL) was added.

Extraction of the derivatives. — A test-tube rack was arranged with columns of tubes, one column for each sample. The first tube in each column was the derivatization-reaction tube, the next three were 16 × 122 mm test tubes, and the final one a 13 × 100 mm culture tube. The second tube contained chloroform (1.0 mL) and water (2.0 mL); the third, water (2.0 mL) and pyridine (0.1 mL); and the

*Parameters designated * may be changed if the computer program is modified as described in the subsection *Changing the integration parameters.*

fourth only water (2.0 mL). The fifth tube was empty. The first reaction mixture was transferred to the second tube (by Pasteur pipette here and for the following transfers), the contents were vortex-mixed (20 sec), and allowed to undergo phase separation while the other derivatives were treated in a similar manner. The lower, chloroform phases were then successively transferred to each of the next tubes, with similar vortex-mixing and phase separation. In the rare case of emulsion formation, the phases were separated by centrifugation. After the final chloroform extracts were transferred to the last tubes, each was condensed to 0.1 mL (70° heating block for 2 h), and the tube was closed with a Teflon-faced screw cap and stored at room temperature.

Gas-liquid chromatographic separation. — The concentrated chloroform extract of the derivatized mixture (1 to 3 μL) was injected on column with a 10 μL Hamilton 701 syringe, equipped with a Chaney adapter. The temperature of the oven was programmed to increase from 140° to 300° at 20°/min, then hold 4 min at 300°, for a total program time of 12 min. The injector was held at 310° and the detector at 320°, and the nitrogen-gas flow was set at 30 mL/min. The columns were of glass, 2 mm (i.d.) × 1.34 m, packed with 3% SP-2250 on 100/120 Supelcoport, sold as Supelco 1-1756. This is an OV-17 on Chromosorb W type of packing. The columns were packed under aspirator vacuum, with light tapping, and were conditioned for 12 h at 240° followed by 2 h at 300°, with nitrogen flow at both temperatures. The flame-ionization detectors on the Varian 6000-series chromatograph were coupled directly to the data processing system.

Recording and processing the data. — The Varian 401 system both plots the chromatogram in real time and stores the raw data set on a floppy disk. The following is a summary of typical commands employed for processing the data presented herein. Analysis parameters: internal standard; by area; stop time, 12 min; expected peaks, 40: equilibrium time, 0; unretained peak time, 0; unidentified peak factor, 0; slice width, 10. Sample parameters: divisor, 50*; amount std., 0.166*; multiplier, 100*. Report parameters: format, E; decimal places, 3; result units, %wetwt. Time-event parameters (all start at time zero): PR, 50000; SN, 100; T%, 5.0; WI, 4; integration inhibit off, 2.5 min. Peak-table parameters: Std. peak #, 1; relative reten. peak #, 1; resolution peak #, 0; resolution minimum, 0; factor %, 25. Identification time windows (±): reference %, 10, min 0; non-reference %, 5, min 0. Response factors, based upon typical column conditions and referenced to L-rhamnose as 1.00, are: D-glucose, 0.96; D-glucitol, 0.54; D-fructose, 1.15; sucrose, 0.84. Detector parameters: range (chromatograph), 11; attenuation (computer), 512; auto-zero, on. Plot parameters: speed, 1 cm/min; plot attenuation, 512.

Calibrating the chromatographic system. — A typical reference standard was made as described under derivatization, with the following changes in the amounts of reactants: L-rhamnose monohydrate (45.9 mg, corresponding to 41.4 mg L-rhamnose), D-glucose (43.2 mg), D-glucitol monohydrate (28.1 mg, corresponding to 25.6 mg of D-glucitol), D-fructose (61.9 mg), sucrose (79.3 mg), and hydroxylamine hydrochloride (189 mg) in pyridine (1.5 mL). For acetylation 1.7 mL of

acetic anhydride was used, and after this step, pyridine (0.3 mL) was added. A scaled extraction sequence was employed, using 5 mL of chloroform in tube 2. An injection (1 μL) into the chromatograph–integrator sysem in the calibration mode, with parameters set as listed in the preceding section, yielded a chromatogram with peak-area counts in the range of two million — corresponding to the peak areas resulting from sugars present at 1% wet weight levels in plant tissue. The septum (blue) was replaced and the column calibrated after every 20 analytical chromatograms.

Changing the integration parameters. — With the use of an internal standard, the amount of derivatized sample injected does not directly affect the calculation of the results. However, the amount injected must be adjusted to yield chromatographic peaks having an area within the region of linear response to each measured sugar. The continuous, analog (A) signal from the detector is converted to a digital (D) format for computation. If the magnitude of the analog signal exceeds the A/D conversion range of the computer, the top of the digitally processed peak will be "clipped". As shown in Fig. 2, an extensive "analog overshoot" can occur without detectable loss of linearity, but when this happens, the parameters marked * in the previous subsections may need to be modified, depending upon the nature of the sample being examined.

Chemicals. — ACS reagent grade pyridine, acetic anhydride, and hydroxylamine hydrochloride were from Fisher Scientific Co., and were used without purification. The saccharides, from commercial sources, were not further purified, but (with the exception of the L-rhamnose and the D-glucitol) were dried in vacuum over phosphorus pentoxide for 24 h at 30° before use.

ACKNOWLEDGMENTS

The authors thank W. Lee Tucker for assistance with the statistical computations. This research was supported, in part, by grant 314845 from the South Dakota State University Research Support Fund.

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