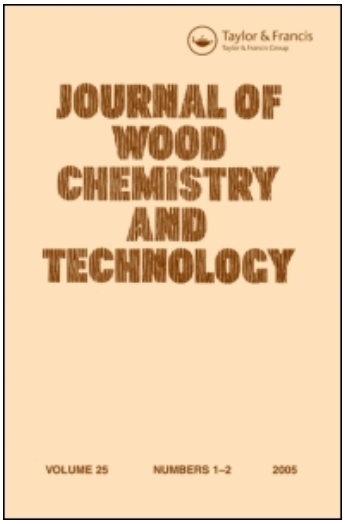

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A Rapid Modified Method for Compositional Carbohydrate Analysis of Lignocellulosics by High pH Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC/PAD)

Mark W. Davis[a]
[a] USDA Forest Service, Forest Products Laboratory, Madison, Wisconsin

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A RAPID MODIFIED METHOD FOR COMPOSITIONAL CARBOHYDRATE ANALYSIS OF LIGNOCELLULOSICS BY HIGH PH ANION-EXCHANGE CHROMATOGRAPHY WITH PULSED AMPEROMETRIC DETECTION (HPAEC/PAD)

Mark W. Davis
USDA Forest Service
Forest Products Laboratory
One Gifford Pinchot Drive
Madison, Wisconsin 53705

ABSTRACT

During the last decade, high pH anion exchange chromatography with pulsed amperometric detection (HPAEC/PAC) has gained increasing acceptance as the method of choice for analysis of neutral sugars commonly occurring in woods, pulps, and other lignocellulosics. This paper describes modified chromatographic conditions and discusses other critical factors that improve the precision and efficiency of this application. The method involves a controlled loading of acetate onto the column prior to equilibration with water and injection of sample. In-line solid-phase extraction is used to remove hydrophobic substances that have the potential to foul the analytical column. Critical operational parameters for the successful application of the method include a metal-free flowpath and a consistent application of anions with sample. Resolution of rhamnose is achieved while maintaining the resolution of xylose and mannose. Simplified sample pretreatment allows a *ca.* five-fold increase in sample through-put compared with gas chromatography of derivatized sugars or to partition chromatography. Run times are less than half those of the widely used hydroxide reverse gradient method for HPAEC/PAD analysis of wood sugars. Long-term system performance data indicate that the method is highly precise and robust. The acetate loading method affords better precision than those of other HPAEC/PAD methods and of gas chromatographic analysis of alditol acetate derivatives by Tappi Method T249 cm-85.

INTRODUCTION

The compositional analysis of lignocellulosics involves acid hydrolysis, typically with sulfuric acid,[1,2] followed by quantitation of the resulting neutral sugar monomers in the hydrolysates by chromatographic means. Alternatively, enzymatic hydrolysis can be employed for the compositional analysis of soluble polysaccharides[3] or in cases wherein the action of enzymes on soluble or insoluble substrates is of interest. Three methods are commonly employed for the chromatographic analysis: (1) gas chromatography (GC) of alditol acetate,[2] trimethylsilyl, or per-*O*-acetylated aldononitrile derivatives,[4] (2) partition chromatography (PC) on cation exchange resins with refractive index detection,[5-7] or (3) high pH anion-exchange chromatography with pulsed amperometric detection (HPAEC/PAD).[3,8-15]

Although results obtained using GC[14] or PC[9] methodologies are comparable with HPAEC/PAD results, the former techniques require multiple sample preparation steps that decrease their efficiency. For sulfuric acid hydrolysates, both methods require neutralization and sulfate removal, typically using barium hydroxide and centrifugation. Subsequent steps for GC analysis typically include reduction with sodium borohydride and derivatization, with attendant concentration and sample clean-up procedures, and finally sample filtration. Subsequent steps for PC analysis include ash removal, concentration, and filtration steps.

In laboratories utilizing HPAEC/PAD, sample preparation varies widely. Some analysts assume it is necessary to neutralize acid hydrolysates[14,16,17] and remove sulfate[8,15,18] prior to chromatographic analysis, and others[9-12] omit these steps. In addition, solid-phase extraction (SPE) is essential for some types of samples[14] and is widely[8,10,15,16] (although not universally)[3,11-13] employed. A major limitation of HPAEC/PAD is the lengthy chromatographic run time, which including column conditioning and re-equilibration steps, is typically 60 min or more.[9-11] Shorter run times have recently been reported,[12] but efforts to reproduce this method have not been successful.[13] A second limitation is the difficulty of resolving the trio of minor hemicellulosic sugars, arabinose, galactose, and rhamnose, while maintaining resolution of the xylose-mannose pair.[10,13,16,17] A third limitation is poor quantitation of low amounts of mannose, because of the common tendency of late eluting peaks to tail excessively.[11,13]

This report describes the operational parameters in use at the USDA Forest Service, Forest Products Laboratory (FPL), which have increased the efficiency and precision of

HPAEC/PAD for the routine analysis of a diverse stream of woods, wood products, and other lignocellulosic samples. Following filtration, sulfuric acid or enzyme hydrolysates are injected directly with no additional sample preparation steps. Matrix hydrophobic components are removed by in-line solid-phase extraction (SPE). A reverse gradient method wherein acetate loading is performed during column conditioning is used to achieve near baseline resolution of the five classic wood sugars and rhamnose. Sugars are eluted within 10 min, with a total run time of 27.5 min. The described method was developed and implemented in June 1994, and has been used to analyze more than 4,000 samples. System performance data collected as part of the FPL quality control program is presented to document the precision and robustness of the method.

EXPERIMENTAL

Materials

$NaC_2H_3O_2 \cdot 3H_2O$ (Ultrapure grade) and carbonate-free NaOH were obtained from J.T. Baker.[1] Water (>17.8 mOhm/cm) was obtained from a point of use polishing system (Barnstead/Thermolyne, Dubuque, IA). Concentrated H_2SO_4 was obtained from EM Science (Gibbstown, NJ). Wood sugar standards and fucose (internal standard) were obtained from Aldrich Chemical Co. (Milwaukee, WI). The wood sugars are arabinose, galactose, rhamnose, glucose, xylose, and mannose. Purities, as certified by certificates of analysis from the vendor, were all in excess of 97.5%. Standard concentrations were determined gravimetrically, with no correction for impurities. Samples of aspen (*Populus tremuloides,* Michx.) and loblolly (*Pinus taeda*, L.), provided by Dr. Masood Ahktar of FPL, were from freshly felled trees that were debarked, chipped, and the chips stored frozen. Kenaf (*Hibiscus cannabinus,* L.) bast was provided by James Han of FPL. The pulp sample, a bleached kraft softwood pulp, was provided by David Bormett of FPL. A large supply of each sample was air dried, milled to pass a 1.00-mm screen, mixed well, and

[1] The use of trade or firm names is for information only and does not imply endorsement by the U.S. Department of Agriculture of any product or service.

stored in sealed polyethylene bags at room temperature for use as quality control performance samples.

Hydrolysis Conditions

Hydrolysis in H_2SO_4 was carried out essentially as described elsewhere.[1,19] Briefly, samples were milled to pass a 1.00-mm screen and vacuum dried at 45°C. Primary hydrolysis of 40-60 mg subsamples was performed with 1.00 mL 72% (w/w) H_2SO_4 for 1 hr at 30°C. Hydrolysates were diluted to 4% (w/w) H_2SO_4 with distilled water, fucose added as an internal standard, and a secondary hydrolysis performed for 1 hr at 120°C. To correct for sugar degradation during secondary hydrolysis, system calibration was based upon a standard mixture of sugars treated in parallel with each batch of samples. Losses during primary hydrolysis are minimal and are ignored.[1] Following filtration through 0.45 μm Teflon syringe filters (National Scientific, Lawrenceville, GA), 5 μL samples of hydrolysates were injected directly onto the chromatographic system with no additional treatment. In some cases, parallel lignin determinations were performed. In these cases, 80-140 mg samples were hydrolyzed, hydrolysates diluted to 100 mL with H_2O, and 10-15 μL portions injected.

Chromatographic Analysis

Sugar contents of hydrolysates were determined by HPAEC/PAD. The chromatographic system consisted of a 738-autosampler (Alcott Chromatography, Norcross, GA), a GPM-1 or a GP40 gradient high pressure pump (Dionex Corp., Sunnyvale, CA), and a pulsed amperometric detector (PAD) (Dionex). The entire flowpath of this system was metal free with the exception of the injection needle (titanium alloy) and injection valve (Hastelloy C). Hydrophobic materials were removed by in-line SPE, employing an NG1 guard column (Dionex) plumbed between the injection valve and Carbo-Pac columns. A time-programmed valve switching event diverted flow around the NG1 column and autosampler 1 min after sample injection. The NG1 guard column was washed with methanol and re-equilibrated with H_2O after every *ca.* 100 injections. Sugar

separation was achieved with Carbo-Pac PA1 guard and analytical columns (Dionex) connected in series. Eluent flow rate was 1.2 mL/min and the temperature was 22°C. The reverse gradient method consisted of elution with H_2O for 11 min, followed by a 1-min ramp to 170 mM $NaC_2H_3O_2$ in 200 mM NaOH, which was maintained for 5 min, then a 1 min return to the original H_2O eluent 9.5 min prior to the next injection. These conditions were employed to elute neutral sugars, condition the column, and re-equilibrate the column, respectively. The time of equilibration is a critical method parameter; therefore, data from the first run of the day were not used. Sugars were detected by their oxidation at a gold electrode surface of the PAD. PAD settings were E1 = 0.1 V, E2 = 0.9 V, and E3 = -0.6 V for durations of 300, 120, and 300 msec, respectively. Output range was set to 10,000 nA/V. To facilitate the pH sensitive oxidation of carbohydrates, 0.30 M NaOH was added to the post-column eluent stream at a flow rate of *ca.* 0.3 mL/min. Detector output was digitized by a Series 900 analog interface (PE Nelson, Cupertino, CA) at a sampling rate of one point/sec and stored and analyzed using Apex ver. 3.14 software (Autochrome, Inc., Milford, MA).

Analysis of Performance Data

System performance (HPAEC/PAD) was monitored by the performance of sugar standards. Standard mixtures, whose sugar concentrations were similar to those of typical sample hydrolysates (Tables 1 and 2), were subjected to secondary hydrolysis in parallel with each hydrolysis batch (typically 18 to 24 samples) and intermittently injected (typically 6 to 8 repetitions) during HPAEC/PAD analysis of each batch. Because the measure of interest in this analysis is the mass of the anhydrous sugar unit as it exists in the sample, all concentrations are given in terms of the anhydrous weight equivalent of the free sugar (*e.g.*, gravimetric mass of glucose is adjusted by 162/180).

Chromatographic performance was assessed by the standard deviations (SDs) of retention times and resolution factors among all standard injections within a period during which the system configuration was not changed. Resolution factors were calculated as the ratio of two times the difference in component retention times with the sum of their peak widths at baseline, or $2\ (RT_2 - RT_1) / (W_1 + W_2)$. System noise was calculated as three times the SD around the chromatographic baseline, as determined by regression

TABLE 1
Relative Response Factor Repeatabilities of Acetate and Hydroxide Reverse Gradient Methods

Analyte	mg/L[a]	RRF Repeatability[b] NaOH[c]	RRF Repeatability[b] Acetate[d]
Arabinose	59.3	1.43	0.94
Galactose	54.4	1.70	1.13
Glucose	697.2	1.20	0.77
Xylose	249.6	1.44	0.89
Mannose	111.1	1.78	1.20

[a]Concentrations here and in all subsequent tables are given in terms of the anhydrous residue of each sugar. Components are listed in order of elution.
[b]To facilitate comparisons between analytes whose concentrations differ, repeatability data are given in terms of relative standard deviations (RSDs). Data are averages of the RSDs among the relative response factors obtained by replicate injections of each analytical batch's standard mixture during routine operation. See Experimental section.
[c]Average RRF RSD from 21 sets of replicate injections performed between January 17 and June 7, 1994.
[d]Average RRF RSD from 27 sets of replicate injections performed between June 16 and November 22, 1994.

analysis in a user-defined area of the chromatogram lacking peaks. Detection limits (LDCs) were then calculated as system noise divided by the ratio of peak height to analyte concentration.

The robustness of a chromatographic method is defined as its continued stability in the context of unavoidable minor fluctuations in operating conditions. The robustness of the acetate loading method was documented by long-term assessments of the repeatability of the peak area determinations upon which analyte quantitations are based. Standard response factors (RFs) were calculated as the chromatographic peak area per unit concentration of each sugar. The relative response factor (RRF) of each analyte was then calculated as the ratio of each analyte's RF to that of the internal standard, fucose. RFs and RRFs are used in calculations of sample analyte concentrations by external standard (ESTD) and internal standard (ISTD) calibration, respectively. Due to the potential for variable sugar degradation during hydrolysis, RFs and RRFs from different hydrolysis batches are not directly comparable. In addition, detector response is subject to variation between HPAEC/PAD batches. Therefore, system repeatability was calculated on a per batch basis as the relative standard deviation (RSD) among the RFs and RRFs of the standard injections interspersed with each batch of samples. Average

TABLE 2
Long-term Relative Response Factor and Response Factor Repeatabilities of the Acetate Reverse Gradient Method

		Repeatability [a]	
Analyte	mg/L	RRF	RF
Arabinose	62.5	1.22	1.86
Galactose	73.0	1.39	1.97
Rhamnose	33.3	2.50	3.18
Glucose	780.0	0.71	1.38
Xylose	356.9	0.81	1.42
Mannose	232.7	0.92	1.47

[a]Repeatabilities calculated as described in Table 1 and Experimental section. RRF and RF RSD values were calculated from the same 139 sets of replicate injections performed between November 22, 1994, and April 1, 1997. RRF and RF values are used for quantitation of sample components by ISTD and ESTD calibration methods, respectively.

system repeatability was then calculated as the mean of the RSDs of these data sets. RSDs were used to facilitate comparisons between the performance of different analytes whose concentrations differed.

The performance of the entire method (hydrolysis plus HPAEC/PAD) was monitored by the hydrolysis and analysis of quality control aspen, loblolly, kenaf, and/or pulp samples with each sample batch. The carbohydrate content of these samples was calculated in terms of percentage dry weight of the original sample, using a one-point internal standard calibration and the anhydrous weight equivalent of each sugar. To facilitate comparisons with other published accounts, inter-batch method repeatabilities are presented as the SDs among sample carbohydrate contents. Because Tappi repeatability is defined as a limit within which agreement is expected 95% of the time between two tests,[20] method SDs were multiplied by 1.96 for comparison with repeatabilities given in Tappi Method T 249 cm-85.[2]

RESULTS AND DISCUSSION

Acetate Loading

Prior to implementation of the acetate-loading method, wood sugar analyses at FPL

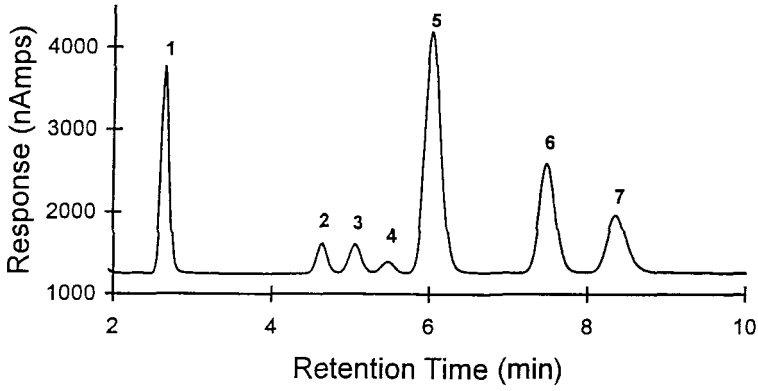

FIGURE 1. HPAEC/PAD chromatogram of a standard mixture of the six neutral sugars commonly found in lignocellulosics. Sugar concentrations are given in Table 2. The ordinate scale represents 35% of the detector's linear range. Peaks 1-7 are fucose, arabinose, galactose, rhamnose, glucose, xylose, and mannose, respectively.

used 300 mM NaOH as conditioning eluent.[9] Under these conditions, arabinose and rhamnose were not reliably resolved and the mannose peak tended to tail. Review of the literature suggests that the former[10,13,16,17] and the latter[11,13] phenomena are widely observed. Efforts were thus undertaken to improve the method.

The acetate loading method described herein involves a 5-min column conditioning step with acetate-hydroxide, followed by column equilibration with water and injection and elution of neutral sugars (see Experimental). The 5-min duration of the conditioning step was chosen to allow elution from the column of uronic acids during the acetate loading. A representative chromatogram obtained by use of the acetate-hydroxide reverse gradient method with a standard mixture is shown in Fig. 1. Excellent resolution of the six wood sugars and the internal standard fucose is achieved within 10 min. With column conditioning and re-equilibration steps included, the total run time of the method is 27.5 min. In our laboratory, use of this method allows the analysis of *ca.* 100 samples and standards in a single chromatographic batch.

In addition to increasing sample through-put, the shorter retention times result in sharper peaks with improved signal-to-noise characteristics. The chromatograms shown

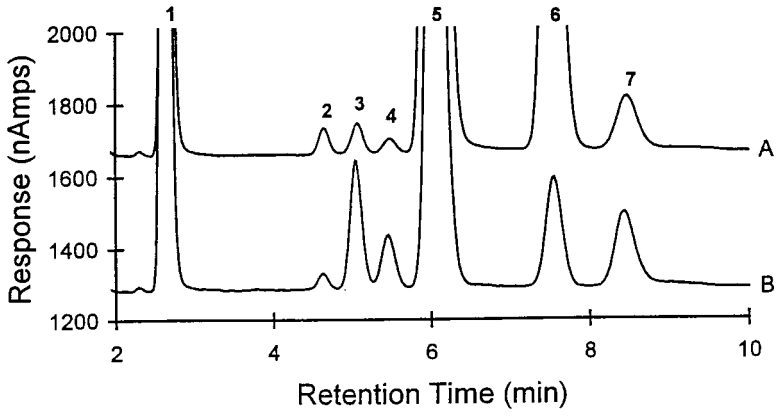

FIGURE 2. HPAEC/PAD chromatograms of sample acid hydrolysates. To facilitate observation of the method's performance with respect to minor sugars, the ordinate scale represents 8% of the detector's linear range. Peak identities are as given in Figure 1. Plot A is derived from aspen wood, plot B from peat moss.

in Fig. 2 were obtained from sample acid hydrolysates, with the ordinate axis reduced to demonstrate the performance of the system with regard to minor sample components. In the case of Fig. 2A, arabinose, galactose, and rhamnose represent 0.39%, 0.54%, and 0.27%, respectively, of the mass of aspen wood. The preponderance of rhamnose compared with arabinose in peat moss (Fig. 2B) illustrates the potential for error inherent in previously reported methods in which arabinose and rhamnose coelute.[9,10,17]

The decrease in retention time by acetate loading was an expected outcome of the original method development work, but the favorable outcome with regard to rhamnose resolution was serendipitous. The acetate-induced shift in retention times turned out to be proportional for all the analytes of interest except rhamnose, for which the effect was less pronounced. Following this observation, it was a simple matter to adjust the acetate loading level to achieve elution of rhamnose between galactose and glucose. Thus, the retention time of rhamnose relative to the other sugars is increased when the level of acetate in the conditioning eluent is increased. This principle can be used to fine-tune chromatographic performance when uncontrolled variables, such as temperature, sample

matrix, or eluent preparation, adversely affect the resolution of rhamnose.

Carbonate loading has recently been reported[12] to provide similar decreases in retention times as those described here. The performance of rhamnose in this system was not described. Although this method has apparently been used successfully for some time by these researchers,[3] efforts to reproduce it in a different laboratory were unsuccessful.[13] A possible explanation for the discrepancy is the strategy used to load the carbonate: Following a brief rinse with hydroxide and water, carbonate was introduced by a 0.1-min elution with a 0.1 N solution. In this case, small differences in the timing of proportioning valve events between different solvent delivery systems could lead to large differences in the amount of carbonate loaded, with a resulting loss in method robustness. Coelution with pusher anion and hydroxide, employed herein, is much less demanding from a system performance standpoint, and provides the added benefit of removing uronic acids from the column prior to the next injection. In addition, a more even distribution of anions on the solid phase likely results from the coelution procedure. It should also be mentioned that operating temperature, a critical parameter,[21] is not specified in either report describing carbonate loading.[12,13] The use of carbonate has not been explored at FPL, although the compatibility of this pusher anion as well as nitrate and sulfate with HPAEC/PAD has been reported.[21] Substitution of sulfate for acetate, albeit at a substantially lower concentration because of sulfate's much greater affinity for the resin, does give a separation very similar to that achieved by acetate loading (data not shown).

Performance Evaluation of the Acetate Loading Method

The extended linear range of the PAD detector makes single-point calibration feasible for wood sugar analyses, which in turn makes the frequent interspersed injection of standard a practical procedure. Using 5 μL injections of standard mixtures, linearity was established up to 1.94 and 1.42 g/L (anhydrous) for glucose and xylose, respectively. Linearity at the lower end is set by detection limits (LDCs), as defined by signal to noise ratios. The detector noise of the system, as described in the Experimental section, is normally under 4 nAmps (0.04% full-scale). Under these conditions, LDCs range from 0.57 to 1.20 mg/L (anhydrous) for 5 μL injections of arabinose and mannose, respectively. These values are in good general agreement with those published for other HPAEC/PAD methods.[3,9,14,22]

The performance of the acetate loading method was compared with that of the hydroxide reverse gradient method by assessing the RRF repeatability exhibited by both methods using the same standard mixture. The standard mixture was composed of the five classic wood sugars at concentrations similar to those typically encountered in sample hydrolysates and fucose as internal standard in 4% (w/w) sulfuric acid. Rhamnose was not included in the standard mixture as it was not resolved by the hydroxide method. Results, compiled during 5 months of routine operation using either method, indicated an improvement in precision of *ca.* 35% associated with the acetate loading method (Table 1). In addition, compositions of quality control samples as determined by either method were indistinguishable (data not shown).

Based on these results, rhamnose was incorporated into the standard mixture and the acetate loading method adopted as the accepted method for the compositional analysis for neutral wood sugars at FPL. The RRF and RF repeatability during the ensuing 2.5 years of operation are shown in Table 2. These data are given as indicators of precisions obtainable by use of either internal standard or external standard calibration methods, respectively. The RRF repeatability of the major sugars glucose, xylose, and mannose was slightly improved compared with the preliminary testing (Table 1). In contrast, a slight decrease in repeatability was observed for arabinose and galactose. In addition, the RRF repeatability of rhamnose, although quite acceptable at 2.5%, was still high relative to those of the other analytes.

This decrease in the RRF repeatability of the minor sugars is likely due to incomplete baseline resolution of these minor sugars from glucose. Although the separation is fairly characterized as near baseline, peak integration typically results in a shared baseline and split peaks for arabinose, galactose, rhamnose, and glucose. Since glucose is present in the standard mixture, as well as in typical sample hydrolysates, at approximately an order of magnitude higher concentration than those of the minor sugars, even small differences in the determination of the point of baseline termination after glucose will have proportionally greater impacts on calculated peak areas of the minor sugars. Rhamnose quantitation, because of the lower concentration and elution between larger peaks characteristic of this analyte, is especially vulnerable. In the preliminary analysis, rhamnose was not included as a standard, and these difficulties were avoided by the establishment of baseline after galactose. Even with the difficulties imposed by introduction of rhamnose as an analyte, the minor sugar RRF repeatabilities obtained with

the acetate loading method represent an improvement over those obtained with the hydroxide method in the absence of rhamnose (Table 1).

Data shown in Table 2 span more than 2 years and a number of substantial changes in system configuration, including but not limited to changes in guard columns, detector cell, and solvent delivery system. The consistency of RRF repeatabilities in the context of change can be taken as evidence of method robustness. The consistent symmetry of the mannose peak is also noteworthy in this regard.

Although analyses in our laboratory are typically performed using fucose as an ISTD, in some cases, fucose is a potential analyte, making this approach impossible. In addition, at times minor sugars are better quantified by injection volumes that place fucose out of the linear range of the detector. In these cases an ESTD analysis is employed. As shown by the RF repeatabilities in Table 2, the sacrifice of precision in such cases is minimal.

Direct assessments of chromatographic performance are shown in Table 3. Here, data were compiled for a shorter duration during which time all system components were held constant, so that chromatographic performance data would be comparable. Retention times were shown to be highly reproducible, with RSDs of <1% for all analytes. A more demanding and relevant test is provided by resolution factors, whose RSDs were within 4% for all analytes. This consistent performance underpins the high degree of precision achieved with the acetate loading method.

Repeatabilities associated with the compositional analysis of lignocellulosic samples are shown in Table 4. These data were compiled from distinct ISTD analyses of distinct quality control subsamples during 2.5 years of routine testing. As such, they represent the inter-batch precision of the entire method, including hydrolysis and HPAEC/PAD analysis. Again, these data sets span a number of system changes, therefore demonstrating the robustness of the method. Error analysis (data not shown) indicates that the bulk of the inter-batch whole method variability is attributable to the intra-batch variability of the chromatography *per se* (Table 2). Thus, these data also demonstrate the robustness of the Saeman hydrolysis procedure.[1]

Precision of the Acetate Loading Method Compared to Other Methods

Comparisons of these data with published accounts of method precision are hampered by the lack of clearly described long-term inter-batch compilations of data. The

TABLE 3
Chromatographic Performance of the Acetate Reverse Gradient Method

Analyte	Retention Time[a]	Resolution Factor[b]
Arabinose	5.01 (0.03)	ND
Galactose	5.41 (0.03)	1.29 (0.02)
Rhamnose	5.79 (0.04)	1.14 (0.04)
Glucose	6.35 (0.05)	1.45 (0.03)
Xylose	7.78 (0.06)	3.27 (0.06)
Mannose	8.55 (0.08)	1.59 (0.04)

[a]Mean (standard deviation) of retention times from 96 injections of standard mixtures performed between May 15 and May 30, 1995. Retention times from operation on different days were directly compared.
[b]Mean (standard deviation) of resolution factors from the same 96 injections as above.

TABLE 4
Carbohydrate Analysis of Quality Control Samples with the Acetate Reverse Gradient Method

Analyte	Aspen[a]	Kenaf[b]	Loblolly[c]	Pulp[d]
Arabinose	0.39 (0.02)	0.46 (0.03)	1.25 (0.04)	0.28 (0.02)
Galactose	0.54 (0.04)	0.97 (0.03)	2.31 (0.09)	0.39 (0.03)
Rhamnose	0.27 (0.03)	0.31 (0.02)	0.10 (0.02)	<0.05
Glucose	45.1 (0.7)	51.4 (0.9)	41.0 (0.6)	82.7 (1.9)
Xylose	15.9 (0.3)	13.0 (0.2)	6.13 (0.10)	5.93 (0.12)
Mannose	1.73 (0.07)	1.26 (0.12)	10.5 (0.2)	6.42 (0.13)

[a]Compositional data for all samples is in terms of mean (standard deviation) percent mass of anhydrous residue per mass sample. Aspen data was obtained by 69 separate analyses on 69 separate occasions between November 22, 1994, and April 1, 1997.
[b]Data obtained by 9 separate analyses between July 8, 1996, and April 1, 1997.
[c]Data obtained by 27 separate analyses between November 1, 1994, and April 1, 1997.
[d]Data obtained by 17 separate analyses between November 1, 1994, and April 23, 1996.

chromatographic variation among intra-batch determinations, wherein all members of the data set are quantified by reference to the same set of standard injections, is subject only to the variability inherent in determination of the relative responses of the samples. Studies that include sample compositional precision data were either explicitly intra-batch in design[9,14] or consistent with an intra-batch design.[10,13] In contrast to intra-batch studies, data sets that include samples and standards hydrolyzed and analyzed on different days are subject to variability introduced by determination of standard RRFs, *i.e.* by the recalibration process. In spite of this, the inter-batch repeatabilities shown in Table 4, in all but a few cases for which they are similar, represent improvements, often substantial, in

the precisions cited by these intra-batch studies.[9,10,13,14]

In a recent report,[17] Puls and coworkers assert that the accuracy of HPAEC/PAD is overestimated and that Borate-HPLC is more reliable. The reader is referred to this source for background on Borate-HPLC, which is not widely used. However, the HPAEC/PAD method upon which they base this conclusion exhibits poor resolution of xylose and mannose, in contrast to the near baseline performance reported for similar methods.[9-11,14] The most straightforward precision data is given in Table 2 of Puls *et al.*,[16] presumably from intra-batch assessments of Norway spruce and beech wood. Because SDs are expected to vary with means, these data sets were compared with sets whose concentrations were similar: loblolly and aspen, respectively (Table 4). Inter-batch precisions obtained by use of the acetate loading method improve on the precisions reported by Puls *et al.* for HPAEC/PAD by an average of 3.5-fold, and on those reported for Borate-HPLC by an average of 1.6-fold. Based on this limited data, HPAEC/PAD would seem to compare favorably with Borate-HPLC.

GC analysis of alditol acetate derivatives remains the recognized Tappi method for determination of carbohydrate composition of woods and pulps,[2] and is therefore still widely used. GC precision data from a long-term repeatability study, analogous to the data sets shown in Table 4, are given for a kraft pulp in section 12.3 of the Tappi Method. In all but one case, in which repeatability was equal, out of the twenty comparisons possible (5 analytes, 4 samples), the precisions exhibited by the acetate loading method represent improvements over those reported in T 249 cm-85.[2] The Table 4 sample whose composition most closely resembles that of the Tappi sample is loblolly pine, whose repeatabilities for arabinose, galactose, glucose, xylose, and mannose are 53%, 20%, 32%, 34%, and 55% those of the Tappi sample. Thus, in addition to providing a many-fold increase in sample through-put, the acetate loading method affords a substantial increase in precision as compared to Tappi Method T 249 cm-85. This finding has recently been substantiated by an intra-batch inter-laboratory round robin study in which FPL participated (data not shown).

In-Line Solid-Phase Extraction

Off-line SPE is widely used to remove soluble lignin and other hydrophobic components that may negatively affect chromatographic performance, and was used

routinely at FPL for some time. However, this technique is time-consuming, and its use is problematic when sample volumes are limited, especially if an appropriate internal standard is not available. Accordingly, an in-line SPE system using a nonmodified divinylbenzene resin (see Experimental) was developed. Prior to implementation of this scheme, it was demonstrated that RFs of sugar standards in the presence and absence of the in-line system were indistinguishable, and that carbohydrate contents of samples as measured with the in-line system were in agreement with those obtained by off-line SPE in its absence (data not shown). In addition, the retention of UV-absorbing material by the NG1 guard was demonstrated by their elution and detection during routine methanol washes of the column. The efficacy of in-line SPE, implemented on January 17, 1994, is demonstrated by the long-term stability of chromatographic results (Table 4) in the context of a diverse sample stream, including sample matrices containing high levels of phenolics and protein. However, the necessity of SPE *per se* is not demonstrated by this finding. It is recommended by the vendor,[15] and should ensure against nonreversible binding of hydrophobics to the divinylbenzene backbone of the CarboPac PA1. However, the stability of chromatography in its absence has been reported for certain sample streams.[3]

Importance of a Metal-Free Flowpath

An additional, and perhaps more significant, benefit of the in-line SPE procedure is that it removes the autosampler, with its titanium alloy needle, from the flowpath for all but *ca.* 3.5 min of aqueous elution during the injection cycle. This is of benefit due to the adverse effect, particularly with regard to mannose and other late-eluting components, that titanium alloys have on chromatographic performance. This observation was made during efforts in 1994 to implement the hydroxide reverse gradient method on a titanium system. During the course of repeated analyses, the tailing of mannose became progressively more pronounced, until the mannose peak was no longer perceptible. Neither acid nor alkali washes were effective in restoring performance with the titanium flowpath, but performance could be restored by an acid wash through a polyetheretherketone (PEEK) flowpath. The same phenomenon, albeit more subtle, was noted with the hydroxide method as implemented on the current system, whose only titanium occurs in the form of the injection needle. Removal of the autosampler from the flowpath after injection prevented the progressive development of tailing of mannose. This

practice has been continued as part of the on-line SPE procedure with the acetate loading method. These observations are consistent with the leaching, stimulated by alkali, from titanium alloy of some presumably anionic component that reversibly binds to the CarboPac resin and adversely affects its chromatographic properties. Interestingly, even though titanium is less subject to leaching on a mass basis than is stainless steel, older equipment with an extensive 316 stainless flowpath was long used successfully with the hydroxide method.

Importance of the Sample Anion Load

The necessity of caution with regard to inorganic anions in the sample matrix is addressed in the vendor literature. In particular, removal of sulfate is mentioned.[8,15] Acid hydrolysates of lignocellulosics, containing 1.2% to 4.0% H_2SO_4 (w/w), constitute the most common sample matrix group encountered at FPL. These are analyzed by direct injection with no prior neutralization or removal of sulfate. In this case, chromatographic performance varies as a function of the amount of sulfate injected with the sample (sample sulfate load, SSL), such that an increase in SSL decreases retention times. The effect is reversible, with a subsequent decrease in SSL shifting retention times back to their original values. This phenomenon exhibits a memory effect, such that the complete retention time shift is not realized until several injections at the new SSL have been made. Apparently, sulfate introduced by sample injection is not completely removed by the conditioning step of the acetate reverse gradient, such that the sulfate load and concomitant retention time changes require several injection cycles to reach their new steady state values. The stable retention times achieved by continuous operation at a given SSL are thus seen as due to the establishment of steady state column-bound concentrations of hydroxide, acetate and sulfate. For this reason, care should be taken to match the SSL for a given analytical batch. Because peaks also become sharper with an increase in SSL, resolution factors are generally slightly improved with 5 μL injections of 4% H_2SO_4 (2.09 μmoles) compared with aqueous samples. The chromatograms shown in the figures and all tabulated data were obtained by injections of within a factor of 2 of this standard SSL. This is apparently sufficient control of SSL for routine operation. Presumably, sample matrices containing other anions, such as buffer salts in the case of enzyme hydrolysates, would give rise to analogous effects.

CONCLUSIONS

This report describes a modified method for the compositional analysis of carbohydrates by HPAEC/PAD that is rapid, precise, and robust. The column is loaded with acetate by elution with 170 mM $NaC_2H_3O_2$ in 200 mM NaOH prior to its equilibration with water and injection of the sample. Robust performance requires that a metal-free flowpath be employed and that anions introduced with the sample be controlled. At FPL, a sample sulfate load of 1-2 μmoles produces satisfactory results.

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