

CARBOHYDRATE ANALYSIS OF PULPS USING ENZYMATIC HYDROLYSIS AND HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

A revised method for analysis of carbohydrates in pulps is presented. The method utilizes commercially available enzyme preparations for enzymatic hydrolysis of the pulp, followed by acid hydrolysis for complete cleavage of oligomers that are still present. Quantification of monosaccharides in the hydrolysate was performed by HPLC using a Pb-sulphonate/polymer column and deionized water as the mobile phase. Integration of the chromatograms was performed using least squares fitting of partially resolved peaks by commercial software. The method requires moderate operator skills and low investment costs for specialized analytical equipment.

INTRODUCTION

Carbohydrate composition of pulps is an increasingly important parameter for evaluating properties that enhance pulp yield and pulp properties. Standardized methods for carbohydrate analysis of pulp involve

acidic hydrolysis of the pulps by sulphuric acid followed by chemical derivatization and subsequent GC analysis¹. It is well known that acidic hydrolysis of polysaccharides in a heterogeneous system is a method that easily introduces non-controllable hydrolysis yield losses with a loss of accuracy in the determination of relative carbohydrate content of pulps². Also, the chemical derivatization required for GC analysis of sugar residues requires operator skills and may introduce additional loss of accuracy in the analysis. Trifluoroacetic acid (TFA) hydrolysis is considered to be a more suitable method for hydrolysing polysaccharides without excessive monosaccharide degradation compared to sulphuric acid hydrolysis³.

Recently, an improved method for carbohydrate analysis of chemical pulps has been published. This method involves the use of enzymes for depolymerization of the pulp⁴⁻⁶. The specialized HPAEC analysis system is expensive as it includes a dedicated PAD detector and post-column modification of the sample. This method has been further developed in our laboratory, using different hydrolysis conditions and a simple HPLC system involving a Pb-sulphonate/polymer column packing material and water as the mobile phase.

This column system has the advantage that oligomers are easily detected as they elute at a lower retention time than any of the relevant monomeric sugars⁷. This approach has the advantage of using less specialized analysis equipment and may thus be useful for laboratories without the sufficient financial resources to obtain equipment that is dedicated to carbohydrate analysis. The disadvantage of this column system is incomplete separation of glucose and xylose. However, the near-baseline separation of these two sugars is sufficient to obtain good accuracy in the integration of the chromatogram, if the chromatographic peaks are resolved mathematically using data analysis.

The paper describes the advantages and limitations of the method. A number of industrial pulps including sulphite, bisulphite and kraft pulps have been analysed.

Table 1 Pulps used in the study

Pulp	Pulp type	Raw material	Kappa
Mg-bisulphite	Industrial	Softwood (mixed <i>Pinus sylvestris</i> and <i>Picea abies</i>) with approximately 10% hardwood	31.7
Mg-bisulphite	Industrial	Hardwood (mixed <i>Betula verrucosa</i> and <i>Populus tremula</i>) with approximately 10% softwood	20.5
Ca-sulphite	Laboratory	Norway spruce (<i>Picea abies</i>)	42.6
Ca-sulphite	Laboratory	Norway spruce (<i>Picea abies</i>)	43.8
ITC kraft	Laboratory	Norway spruce (<i>Picea abies</i>)	32.6
Conventional PS/AQ kraft	Laboratory	Norway spruce (<i>Picea abies</i>)	32.0
ITC PS/AQ kraft	Laboratory	Norway spruce (<i>Picea abies</i>)	30.1

EXPERIMENTAL

Pulps

Seven different pulps were studied. These include laboratory ITC kraft softwood pulp, laboratory high-yield polysulphide/AQ (PS/AQ) pulps, industrial Mg-bisulphite softwood and hardwood pulps and laboratory Ca-sulphite softwood pulps. Properties of the pulps are given in Table 1. Conditions for manufacturing the kraft pulps are reported elsewhere⁸. The kappa numbers of the pulps were determined according to SCAN-C 1:77 and the lignin content of the pulps (% on weight) were calculated as $0.15 \cdot \text{kappa}$ for the kraft pulps and $0.17 \cdot \text{kappa}$ for the sulphite pulps⁹.

Hydrolysis of the pulps

Enzymatic hydrolysis was done by using 5 g o.d. pulp. The pulp was mixed with 25 ml acetic acid buffer (pH=5, 1M solution) and 10 ml of 100g/l mannitol solution as an internal standard. Deionized water was added to a total weight of 250g. The samples were incubated at 55 °C for 24 hours.

A mixture of equal amounts of commercial cellulase (ECOPULP[®] C-15), xylanase (ECOPULP[®] TX-200 C) and mannase (KMB 800103) solutions

from Röhme Enzyme Finland Oy was used. 3 ml of the enzyme mixture was added after 0, 3 and 6 hours. After enzymatic hydrolysis, the total weight of each hydrolysate was brought up to 250 g with deionized water to compensate for evaporation losses.

As enzymatic hydrolysis gives a mixture of mono- and disaccharides and oligomers, a mild acid hydrolysis with 1M trifluoroacetic acid (TFA) was performed in order to get a complete decomposition into monosaccharides. The samples with TFA were hydrolysed in heat-resistant closed bottles in boiling water for one hour. The samples were neutralized to pH 5 by adding about 2.4g Ba(OH)₂ to 25 ml of the hydrolysate. Before injection, the samples were filtered through a 0.22 µm millipore filter. Acid hydrolysis was partially optimized by running samples at different degrees of hydrolysis:

- 1) No TFA hydrolysis
- 2) Single stage TFA hydrolysis (preferred)
- 3) Two-stage TFA hydrolysis

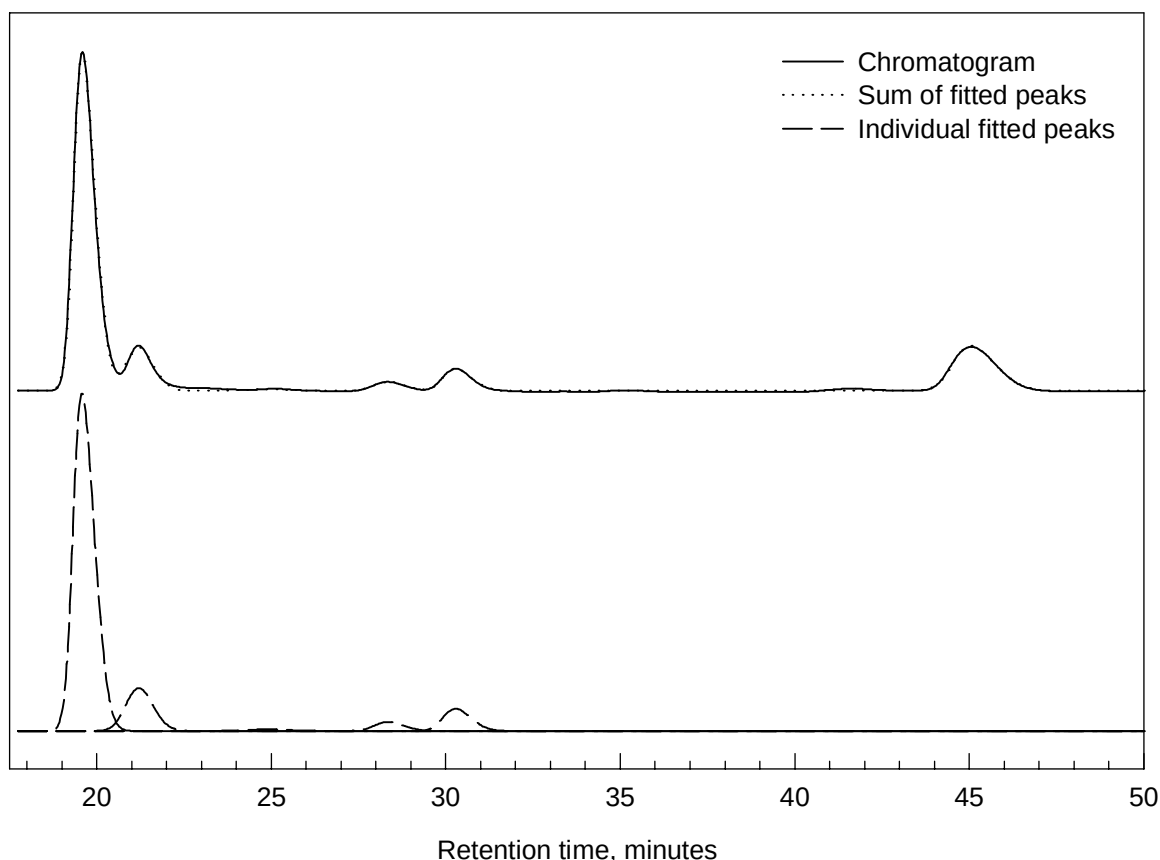


Fig. 1 Least squares fit of chromatogram of Mg-bisulphite hardwood pulp hydrolysate. $r^2 = 0.9994$. The original chromatogram (—) and the sum of the fitted peaks (.....) are overlaid. Individual peaks (- -) are shown at the bottom of the plot.

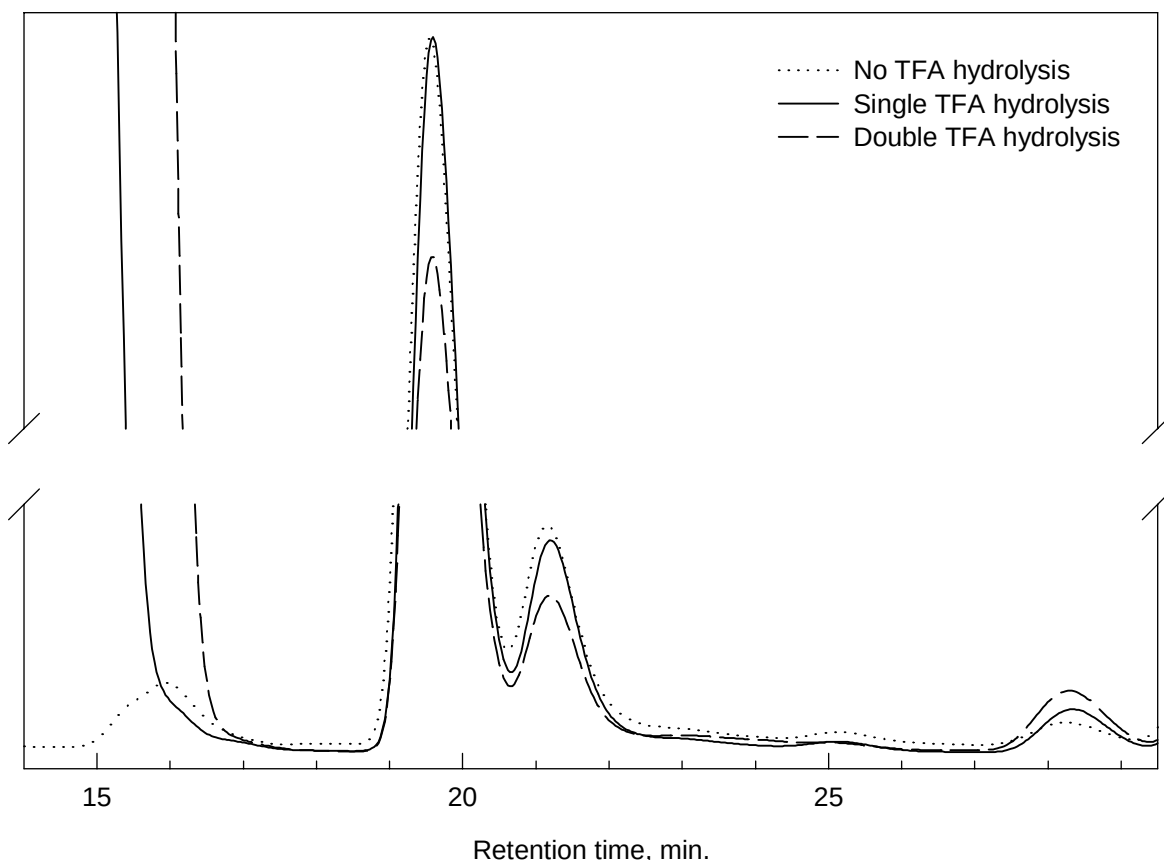


Fig. 2 Chromatogram of hardwood Mg-bisulphite pulp hydrolysed to different degrees. Note the decreased hydrolysis yield of glucose and xylose after double TFA hydrolysis.

HPLC analysis

Chromatographic separation of the hydrolysate was performed on a Chrompack Carbohydrates Pb column using deionized, filtered and degassed water as mobile phase. Column temperature was 80 °C. The HPLC system consisted of a Shimadzu LC-6A pump, manual injector, Shimadzu CTO-10A column oven and Shimadzu RID-6A refractive index detector. The chromatogram was acquired using Shimadzu's CLASS-VP chromatography software, and the chromatogram was exported as an ASCII file for further processing.

Processing and integration of the chromatograms

Since the column system does not give full baseline separation of glucose and xylose, standard integration of the chromatogram using the chromatography software could not give sufficient precision for quantitative determination of the monomer ratios in the hydrolysate. ASCII files containing the data for each chromatogram were generated from the chromatography software and imported into a commercial PC software program (Peakfit from SPSS). Baseline correction and data reduction were performed inside the software, and the chromatogram was fitted to the required number of individual peaks. Peak shape is described by Peakfit's

$$y = \frac{a_0}{a_3} \left[1 - \exp\left(-\frac{a_3}{a_2}\right) \right] \left[\frac{\sqrt{\frac{a_1}{x}} I_1\left(\frac{2\sqrt{a_1 x}}{a_2}\right) \exp\left(\frac{-x-a_1}{a_2}\right)}{1 - T\left(\frac{a_1}{a_2}, \frac{x}{a_2}\right) \left[1 - \exp\left(-\frac{a_3}{a_2}\right) \right]} \right]$$

$$T(u, v) = \exp(-v) \int_0^u \exp(-t) I_0(2\sqrt{vt}) dt$$

Eq. 1

I_0 and I_1 are modified Bessel functions

a_0 =area

a_1 =center

a_2 =width

a_3 =distortion

Non-Linear Chromatography function (Eq. 1). The software provided relative areas for each fitted peak.

Calibration

Standard solutions of D-glucose, D-xylose, D-galactose, L-arabinose and D-mannose in different ratios were prepared. Mannitol was used as an internal standard. The chromatograms were analysed as described.

Calculations

All values for monosaccharide concentrations in the hydrolysate were corrected for the small contribution by the enzyme preparation (Fig. 3).

In calculating the contents of the different polysaccharides of the pulps, a correction factor was introduced to correct for the increased mass of the carbohydrates by the addition of one water molecule for each anhydrosaccharide unit. It was assumed that the glucose:mannose ratio of the glucomannans was 1:3, and that all arabinose was linked to the xylan (Eq. 2)¹⁰.

$$\begin{aligned}\% \text{ Glucomannan} &= \frac{4}{3} \cdot \text{Man} \\ \% \text{ Cellulose} &= \text{Glc} - \frac{1}{3} \cdot \text{Man} \\ \% \text{ Xylan} &= \text{Xyl} + \text{Ara}\end{aligned}\quad \text{Eq. 2}$$

where *Glc*, *Man*, *Xyl* and *Ara* are the relative amounts (in % by weight) of each monosaccharide in the hydrolysate, corrected for the addition of one molecule of water per anhydrosaccharide unit during hydrolysis

RESULTS AND DISCUSSION

HPLC and the analysis of the chromatograms

An example of the separation of pulp carbohydrates is shown in Fig. 1. For the pulp hydrolysates, the column system gives baseline separation of all monosaccharides except for glucose and xylose, which are overlapping slightly. As can be seen from the figure,

the mathematical separation of overlapping peaks in the HPLC chromatogram is very good. The sum of the fitted peaks closely matches the original chromatogram ($0.997 < r^2 < 0.9994$). The column shows good reproducibility and little degradation in performance.

Hydrolysis

Chromatograms from experiments with various hydrolysis conditions are shown in Fig. 2. With no TFA hydrolysis, oligosaccharides can be detected in the hydrolysate, indicating that the hydrolysis is not complete. Very small amounts of oligomers are detected after single-stage TFA hydrolysis. However, the mannose yield increases substantially, indicating that the oligomers are mainly due to incomplete enzymatic hydrolysis of the branched galactoglucomannan. After double-stage TFA hydrolysis, only a smaller change in mannose yield can be observed, but a substantial loss of glucose and xylose is apparent. Further optimization of the TFA hydrolysis procedure therefore seems necessary for optimal yield of the monosaccharides. The hydrolysis yields were in the range of 93-102% (Table 2).

Carbohydrate composition of pulps

Since very few laboratories are able to perform carbohydrate analyses at near 100% hydrolysis yields of monosaccharides, it is difficult to obtain reference data for the pulps studied. However, some general com-

Table 2 Carbohydrate analysis data for different pulps using Pb-sulphonate column and least squares fitting of non-resolved peaks

Pulp	Raw material	Lignin content (%) ^{b)}	Analytical hydrolysis yield (%) ^{c)}	Relative carbohydrate composition (%) ^{a)}		
				Cellulose	Glucomannan	Xylan ^{d)}
Mg-bisulphite ^{e)}	Softwood	5.4	100.2	81.1	8.1	10.7
Mg-bisulphite ^{e)}	Hardwood	3.5	94.4	82.6	2.8	14.6
Ca-sulphite ^{f)}	Softwood	7.2	99.6	79.8	8.3	11.8
Ca-sulphite ^{f)}	Softwood	7.4	100.1	79.8	9.1	11.1
ITC kraft ^{f)}	Softwood	4.9	97.5	81.0	7.8	11.2
Conventional PS/AQ kraft ^{f)}	Softwood	4.8	102.4	73.8	9.8	16.4
ITC PS/AQ kraft ^{f)}	Softwood	4.5	93.4	80.0	9.4	10.7

a) Of lignin-free pulp

b) Kraft pulp lignin content (%) = 0.15·kappa no.
Sulphite pulp lignin content (%) = 0.17·kappa no.

c) % of lignin-free pulp

d) Including arabinan

e) Industrial pulp

f) Lab. pulp

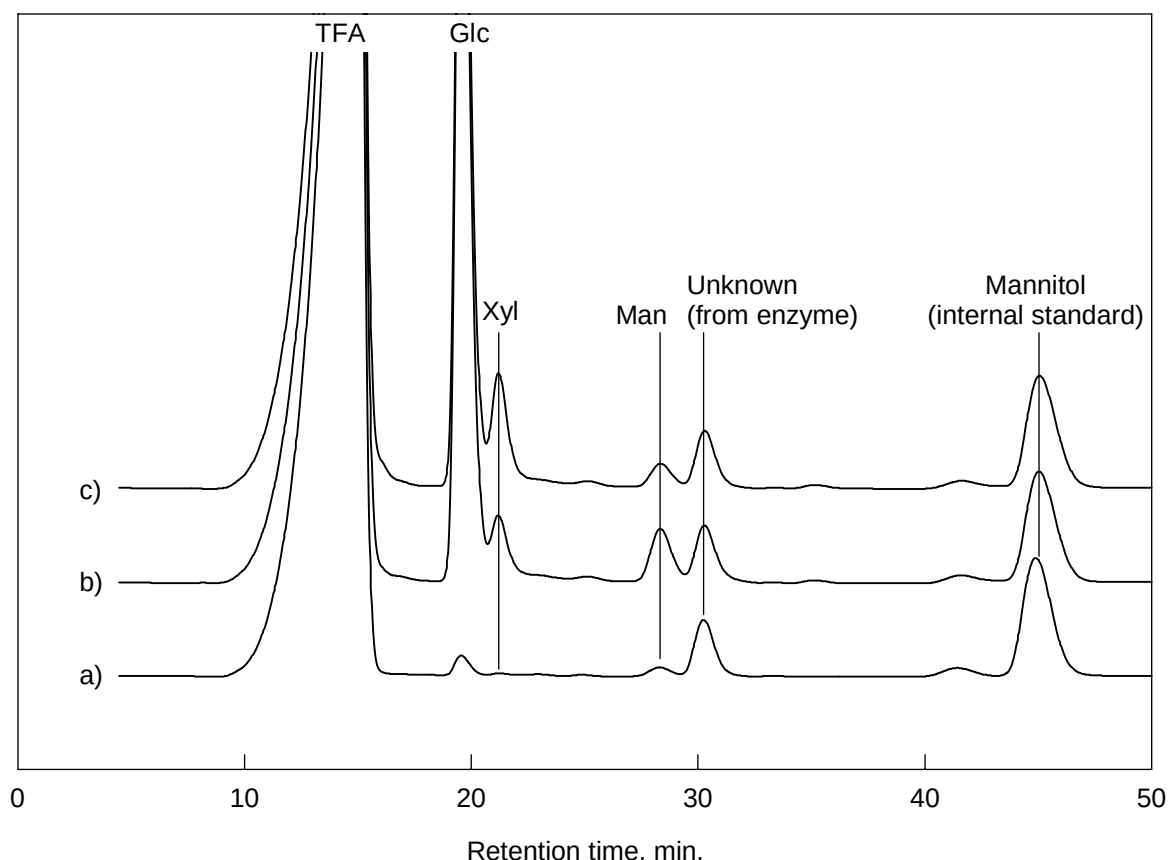


Fig. 3 Chromatogram of industrial Mg-bisulphite pulp hydrolysates. a) Blank, no pulp; b) Softwood pulp; c) Hardwood pulp. See Table 1 for raw material specifications. The different hemicellulose composition of the two pulps is apparent from the chromatogram.

ments can be made on the carbohydrate composition of the pulps. As expected, the two Mg-bisulphite pulps made from different raw material have distinctly different carbohydrate profiles. The well-known composition of hardwood and softwood hemicelluloses (hardwood hemicelluloses containing a high content of xylan, and softwoods hemicelluloses containing predominantly glucomannan) can easily be seen in Table 2 and are also apparent from Fig. 3.

Furthermore, comparing the kraft pulps, the expected increase in hemicellulose content upon addition of polysulphide and AQ to the cooking liquor is observed. The conventional PS/AQ kraft pulp apparently has a very high content of xylan, which is to be expected taking into account the possibility of xylan reprecipitation during the cook. For the ITC PS/AQ kraft pulp, a general yield increase for all carbohydrate components can be observed, in accordance with what is known about the yield-enhancing effects of polysulphide in kraft cooking¹¹.

CONCLUSIONS

Enzymatic hydrolysis followed by trifluoroacetic acid hydrolysis seems to be a useful method for the analysis of carbohydrate composition in pulps. For

maximum yield of monosaccharides, careful optimization of the acid hydrolysis stage is important. The combination of enzymatic and acidic hydrolysis gave yields close to 100%. The enzymatic hydrolysis was performed with commercially available enzymes.

HPLC analysis of the hydrolysate using a Pb-sulphonate stationary phase and water as mobile phase gives good accuracy in the determination of the carbohydrate composition of the hydrolysate, provided integration of partially overlapping peaks is performed with appropriate software. The HPLC analysis does not require specialized equipment apart from an HPLC system equipped with a refractive index detector and an appropriate column, and the method can thus be utilized in laboratories lacking the resources to obtain specialized carbohydrate analysis. Thus, the need for expensive, specialized ion chromatographic column systems and equally specialized detectors is minimized.

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REFERENCES

1. TAPPI Test method no. T249 cm-85
2. Chambers, R.E., Clamp, J.L., *Biochem. J.* 125, 1009 (1971)
3. De Ruiter, G.A., Schols, H.A., Voragen, A.G.J., Rombouts, F.M., *Anal. Biochem.* 207, 176 (1992)
4. Buchert, J., Siika-aho, M., Bailey, M., Valkeajärvi, A., Pere, J., Viikari, L., *Biotechn. Techniques* 7(11), 785 (1993)
5. Hausalo, T., *Proc. 8th ISWPC*, Vol. III, p. 131 (1995)
6. Tenkanen, M., Hausalo, T., Siika-aho, M., Buchert, J., Viikari, L., *Proc. 8th ISWPC*, Vol. III, p. 189 (1995)
7. Chrompack product specification sheet, Carbohydrates Pb column
8. Minja, R.J.A., Moe, S.T., Kleppe, P.J., *TAPPI Breaking the pulp yield barrier symposium*, TAPPI Press, Atlanta, p. 213 (1998)
9. Rydholm, S., *Pulping processes*, Wiley Interscience, New York (1965)
10. Sjöström, E., *Wood Chemistry*, 2nd ed., Academic Press, Inc., San Diego
11. Kleppe, P.J., Kringstad, K., *Norsk Skogindustri* 11, 428 (1963)