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An HPLC method for the quantitative determination of hexeneuronic acid groups in chemical pulps [1]

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Abstract

It has recently been demonstrated that 4-deoxy-L-*threo*-hex-4-enopyranosyluronic acid (''hexeneuronic acid'') is present in kraft pulps and linked to the xylan backbone. An analytical method for the quantitative determination of hexeneuronic acid groups has now been developed. The procedure involves a selective hydrolysis with mercuric acetate of the glucosidic linkage between the hexeneuronic acid group and the xylan chain, followed by oxidation with periodate to form β-formyl pyruvic acid. The latter is reacted with thiobarbituric acid, and the red-coloured adduct formed is separated by reverse phase HPLC and quantified by measuring the absorbance at 549 nm. Some kraft pulps have been analysed to illustrate the contribution of hexeneuronic acid groups to the total amount of oxidizable structures present in such pulps. © 1996 Elsevier Science Ltd.

Keywords: 4-Deoxy-L-*threo*-hex-4-enopyranosyluronic acid; Xylan; Quantitative analysis; Colorimetry; Kraft pulps

1. Introduction

During alkaline pulping of wood, 4-*O*-methyl-D-glucuronic acid groups present in xylan are in part converted into the corresponding unsaturated acid by the loss of methanol (Scheme 1). This reaction was first described by the use of a model compound for xylan, 2-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-xylitol [1]. On treatment of this compound with 1 M sodium hydroxide at 150 °C, a 50%-yield of 2-*O*-(4-deoxy-

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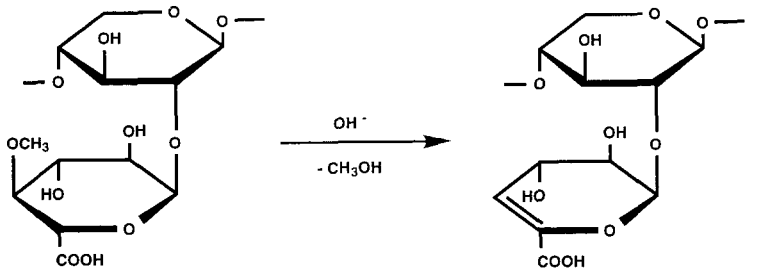


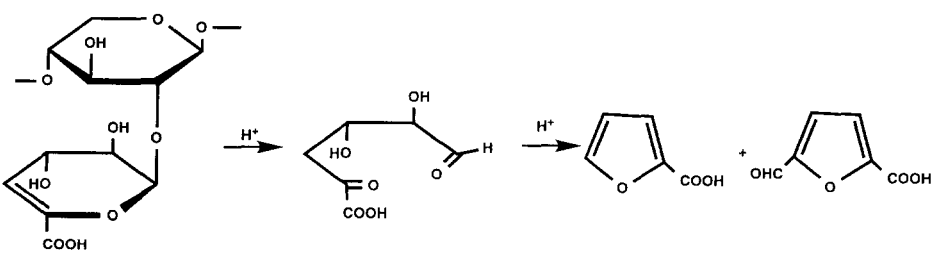
Scheme 1. Formation of hexeneuronic acid groups in xylan during alkaline pulping.

β-L-*threo*-hex-4-enopyranosyluronic acid)-D-xylitol (hexeneuronic acid-D-xylitol) was obtained after 90 min reaction time. When the reaction time was prolonged, the yield decreased. Based on the model study, it was suggested that hexeneuronic acid groups should be present in kraft pulps.

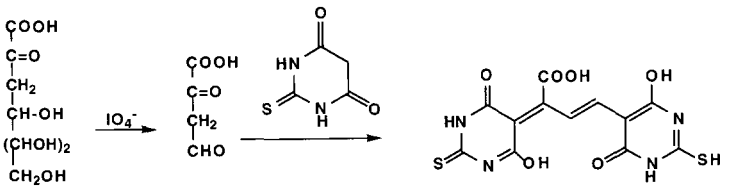
Recently, treatment of pine kraft pulps with xylanase and subsequent analysis by ^{1}H NMR spectroscopy confirmed the presence of hexeneuronic acid groups as part of the pulp xylan [2]. Furthermore, it was demonstrated that these groups are formed early in the kraft cook, i.e. during the heating up period of the wood. Analysis of the dissolved xylan fraction present in kraft cooking liquor showed that this fraction also contained the hexeneuronic acid structure [3].

. The presence of unsaturated structures in pulps which does not originate from lignin is of interest, since such structures will interfere with the method of determination of residual lignin (the kappa number) and may thus contribute to an erroneously high lignin content. Moreover, such structures will have a different reactivity as compared to lignin towards various bleaching agents and they will contribute to a higher demand for bleaching chemicals. Under acidic conditions, the hexeneuronic acid structure is degraded and gives rise to two furan derivatives as depicted in Scheme 2 [4–6]. This may explain why these structures have escaped detection for a long time, since traditional analytical methods for pulp carbohydrates usually involve an acid hydrolysis step.

The quantitative analysis of hexeneuronic acid present in pulps can be achieved, as described above, by enzymatic hydrolysis using xylanase followed by NMR spectroscopy. Alternatively, the enzymatically released hexeneuronic acid structures which are still linked to one or more xylose units can be separated by anion-exchange



Scheme 2. Products from the acid hydrolysis of hexeneuronic acid groups.



Scheme 3. The formation of thiobarbituric acid–β-formyl pyruvate from 2-keto-3-deoxyheptonic acid.

chromatography [7] or by capillary zone electrophoresis [8]. In either case, the overall method is time consuming and, for accessibility reasons, the enzymatic hydrolysis is not capable of releasing more than a portion of the total amount of xylan present in a pulp sample unless a comprehensive enzymatic treatment involving cellulases as well as xylanases is used [2].

The determination of unsaturated uronic acid structures is important in the analysis of connective-tissue polysaccharides, since bacterial and fungal eliminases are highly efficient systems for the creation of such structures. Ludwigs et al. [9] used mercuric salts (chloride or acetate) for the complete hydrolytic release of the unsaturated uronic acid from 2-acetamido-2-deoxy-3-*O*-(β-D-*threo*-4-enopyranosyluronic acid)-D-glucose. The acid was obtained as an equilibrium mixture of the ring-closed enol form and the corresponding open 3-deoxy-2-keto acid. For acids of the latter type, a sensitive colour test has been developed involving a periodate oxidation to β-formyl pyruvic acid and the coupling of the latter with thiobarbituric acid (TBA) to form a chromogen with a light-absorption maximum at 549 nm (Scheme 3) [10]. The method has, e.g., been used for quantitative spectrophotometric thin-layer chromatography of TBA–β-formyl pyruvate [11].

Based on a combination of the principles described in refs [9,10], the objective of the present work has been to develop a simple method for the quantitative analysis of hexeneuronic acids present in pulp samples. Chromatographic separation by HPLC to obtain the pure light-absorbing TBA-adduct, as well as optimization of reaction parameters, was found to be necessary in order to achieve a reproducible and quantitative method.

2. Experimental

Materials and apparatus.—Industrial birch kraft pulps from Swedish mills, and laboratory-made birch and pine kraft pulps, were thoroughly washed with distilled water and air dried. In order to remove all extractives, the pulps were extracted with acetone for 12 h in a Soxhlet extractor. 4,6-Dihydroxy-2-mercaptopyrimidine (thiobarbituric acid, TBA) was from Acros Organics, and 3-deoxyoctulosonic acid ammonium salt (ammonium 3-deoxy-2-keto-octonate) was from Sigma Chemical Co. The HPLC system consisted of two Waters 510 pumps, a Waters Model 996 photo diodearray detector and a NEC Power Mate 433 data processor. Separations were carried out on a Nucleosil 5 C-18 column (4.6 × 200 mm). Methanol and trifluoroacetic acid of HPLC grade were used.

Treatment of pulp with mercuric chloride.—Purified pulp (1 g) was mixed with 30 mL water and allowed to swell at room temperature for 1 h with stirring. An equal vol of 20 mM mercuric chloride was added, and the mixture was heated at 60 °C for 90 min with continuous stirring. The pulp residue was filtered and washed with water, and the filtrate was collected for further treatment as described below.

Treatment of pulp with mercuric acetate.—A pH-5 solution of 70 mM mercuric acetate was made by dissolving 4.84 g of mercuric acetate in 60 mL of 0.2 M AcOH containing 4.66 g of NaOAc and diluting to 250 mL. The pulp sample (1 g) was swollen in 30 mL water as above, and an equal vol of mercuric acetate solution was added. The mixture was kept at room temperature for 20 min with continuous stirring, and, after filtering and washing, the filtrate was collected.

Oxidation with periodate and reaction with TBA.—After hydrolysis, the filtrate volume was adjusted to 60 mL. From this solution, 300 μL were acidified with 60 μL of concentrated HCl and immediately reacted with 0.5 mL of periodate solution (0.1 M sodium metaperiodate in 2.8 M phosphoric acid) during 20 min at room temperature in a test tube. The resulting solution was treated with 2 mL of an arsenite solution (4% sodium arsenite in 0.5 M hydrochloric acid) at room temperature until the brown colour disappeared (approximately 30 min). Thiobarbituric acid (0.3% in water) was added to a total volume of 10 mL and the tube was heated at 50 °C in a water bath for 170 min, followed directly by analysis of the resulting TBA–β-formyl pyruvic acid adduct after a HPLC separation, or (sometimes) by a direct spectrophotometric measurement.

Analysis of the TBA–β-formyl pyruvic acid adduct.—HPLC Separation was done on 100 μL of the TBA-adduct solution using a mobile phase of water containing 0.5% of trifluoroacetic acid for 2 min, followed by a linear gradient of MeOH in water (0–100% in 10 min) containing 0.5% trifluoroacetic acid. The flow rate was 1 mL/min and the TBA-adduct was monitored at 549 nm. In direct measurements of the TBA-adduct, the solution was analysed directly at 549 nm with 1 mL mercuric chloride or mercuric acetate solution treated in the same manner as the sample solution in the reference cuvette.

For quantification, a calibration curve was constructed using ammonium 3-deoxy-2-keto-octonate as a model. This compound (1.0 mg) was dissolved in 1 mL of water, and from the resulting solution 20, 40, 80, and 160-μL samples were taken and each dissolved in 1 mL of 35 mM mercuric acetate. All solutions were subsequently treated with periodate, arsenite, and TBA as described above and analysed for the TBA–β-formyl pyruvic acid adduct.

3. Results and discussion

Hydrolysis.—A sample of birch kraft pulp was subjected to hydrolysis with mercuric chloride at 60 °C for various amounts of time, and the release of hexeneuronic acid was monitored after subsequent periodate oxidation and reaction with TBA. As shown in Fig. 1, the hydrolysis step proceeded quite rapidly, a maximum in absorbance being reached after approximately 50 min. The proposed mechanism involves the addition of a mercuric chloride ion across the vinyl ether double bond followed by hydrolysis to a

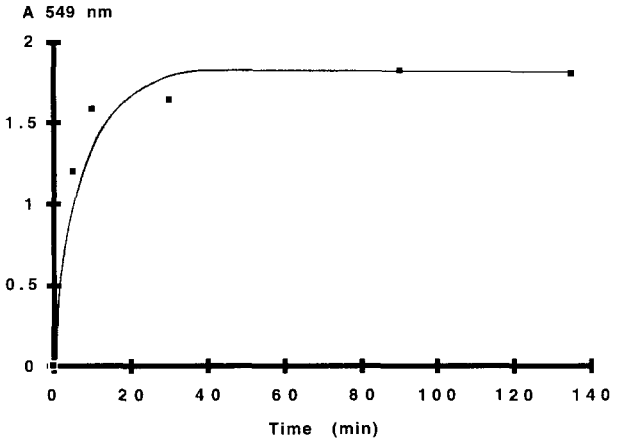
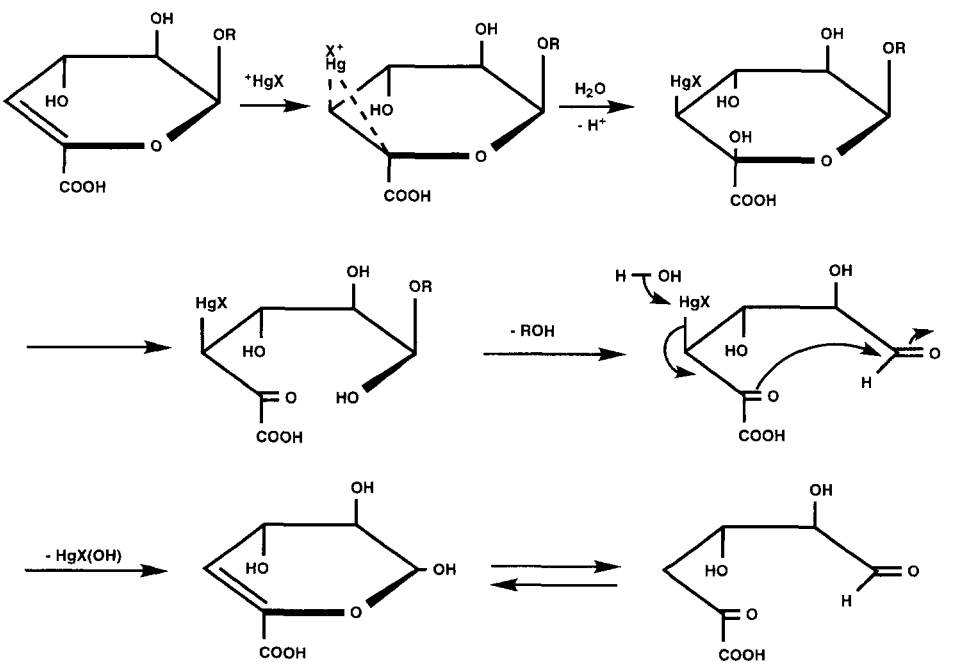


Fig. 1. Influence of reaction time on the release of hexeneuronic acid from pulp xylan by mercuric chloride solution at 60 °C, as determined by TBA.

hemiketal structure. The latter is further hydrolysed with loss of the aglycone and subsequent elimination of mercuric hydroxychloride (Scheme 4).

With a constant reaction time of 90 min, a change in the temperature of the



Scheme 4. Proposed mechanism for the release of hexeneuronic acid from xylan by hydrolysis with aqueous mercuric (II) solution.

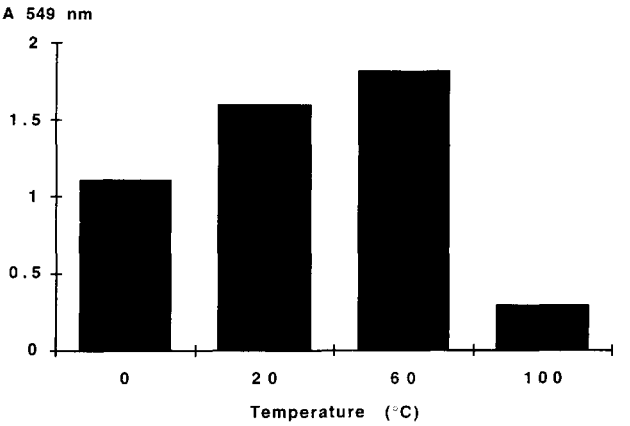


Fig. 2. Influence of temperature on the release of hexeneuronic acid from pulp xylan by mercuric chloride solution, as determined by TBA.

hydrolysis step resulted in an increase in the absorbance at 549 nm in the 0–60 °C range, whereas a higher temperature (100 °C) resulted in a lower absorbance value, as shown in Fig. 2. This indicates a decomposition at high temperature of the 3-deoxy-2-keto-glucuronic acid formed in accordance with reported data on 3-deoxy-2-keto-heptonic acid [10], which show that the latter structure has a half-life of approximately 30–40 min at 100 °C in 1 M hydrochloric acid.

Treatment of hexeneuronic acid with a solution of mercuric chloride may result in a successive degradation into furan derivatives since the solution is strongly acidic. A mercuric salt able to achieve the hydrolytic reaction at a higher pH-value was therefore desirable. Aqueous mercuric acetate, in an acetate buffer, was shown to give a rapid release of hexeneuronic acid under mild conditions, i.e. room temperature and less than 10 min reaction time (Fig. 3). The reason for this is probably that the degree of dissociation of the mercuric acetate is higher than that of the chloride. When the reaction with mercuric acetate was interrupted after 20 min, a subsequent second treatment of the washed pulp with fresh mercuric acetate did not yield any further absorbance at 549 nm, indicating that the reaction had gone to completion in the first treatment.

Oxidation and reaction with TBA.—The periodate oxidation results in an oxidative cleavage of the glycol structure in the hexeneuronic acid to form formyl pyruvic acid. It was found, however, that a direct addition of periodate to the mixture after the mercuric acetate hydrolysis gave a precipitate which interfered with the subsequent reaction steps. This precipitate was not identified, but it could be avoided by adding hydrochloric acid immediately before the addition of periodate.

After the hydrolysis, mercuric (II) ions are present in the solution and these are able to oxidize TBA–β-formyl pyruvic acid, which makes it impossible to obtain accurate colorimetric data. Therefore, prior to the reaction with TBA, the solution must be reduced by the addition of sodium arsenite. This converts all mercuric ions into mercurous (I) ions and, at the same time, all periodate/iodate from the periodate oxidation is reduced to iodide. Unless an excess of iodide ions is present in the solution,

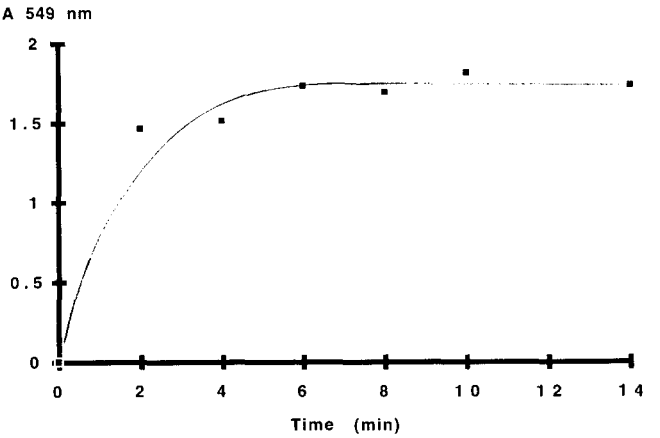


Fig. 3. Influence of reaction time on the release of hexeneuronic acid from pulp xylan by mercuric acetate solution at room temperature, as determined by TBA.

a precipitate of mercurous iodide (Hg_2I_2) forms again, making colorimetric detection impossible. This requirement can be fulfilled either by adding enough periodate in the oxidation step, or by adding a potassium iodide solution after the arsenite reduction in order to convert all mercurous iodide into colourless HgI_4^{2-}.

According to the literature, the reaction between TBA and the periodate oxidation product from 3-deoxy-2-keto sugar acids should be completely specific and should give a chromogen having an absorption maximum at 550 nm. However, when the TBA-reaction was performed with commercial ammonium 3-deoxy-2-keto-octonate at 100 °C, a sharp maximum in colour formation was observed after about 6 min. At longer heating

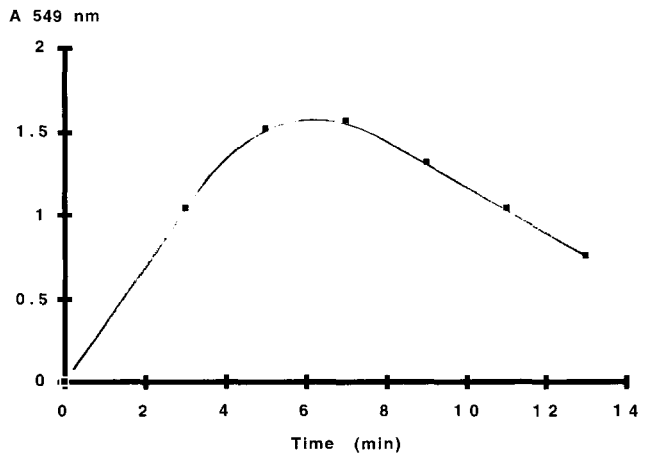


Fig. 4. Influence of reaction time at 100 °C on the reaction between TBA and β-formyl pyruvic acid from ammonium 3-deoxy-2-keto-octonate, as monitored at 549 nm.

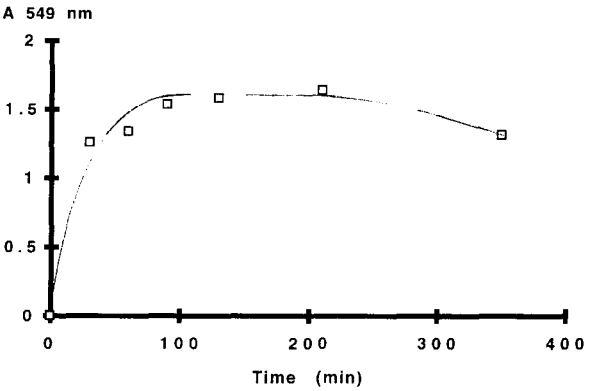


Fig. 5. Influence of reaction time at 50 °C on the reaction between TBA and β-formyl pyruvic acid from ammonium 3-deoxy-2-keto-octonate, as monitored at 549 nm.

times, the absorption intensity decreased as shown in Fig. 4. In addition, it was found that TBA itself when heated under acidic conditions decomposes into coloured structures [12]. Thus, several difficulties were encountered in finding conditions under which both the reference and the sample solution were stable enough to permit accurate readings of molar extinction coefficients, difficulties which may explain the differences in extinction coefficients reported in the literature [10,13].

In order to optimise the conditions for the formation of the coloured addition compound between TBA and β-formyl pyruvic acid, ammonium 3-deoxy-2-keto-octonate was used as a model compound. On this, the oxidation–reduction–TBA-reaction was run and the formation of colour at 549 nm was monitored as a function of time at 50 °C and at 25 °C, respectively. A maximum in absorbance intensity was found after approximately 130 min when the reaction was run at 50 °C, and this intensity remained for more than 1 h. At very long reaction times, a slow decomposition of the reaction product was noticed (Fig. 5). The molar extinction coefficient of TBA–β-formyl pyruvic acid was found to be 49,000, which is somewhat higher than the value reported in ref. [10] (44,000) and much larger than the value in ref. [13] (17,380). In the reaction at room temperature, a maximum in absorbance was found after 21 h giving the same extinction coefficient.

HPLC Separation.—The structure and properties of the adduct between TBA and β-formyl pyruvic acid have been described, and the very good stability of the compound in acidic and neutral aqueous solutions was noted [14]. Separation by HPLC of the reaction mixture containing TBA, inorganic ions, and the reaction product showed two peaks at 10.6 and 11.2 min, the latter representing the actual TBA-adduct with an absorption maximum at 549 nm. The other peak corresponds to a decomposition product from TBA–β-formyl pyruvic acid having an absorption maximum at 530 nm. Unless a chromatographic separation is carried out, these two products interfere with each other giving an overlap of absorbancies which makes it difficult to get quantitative calculations.

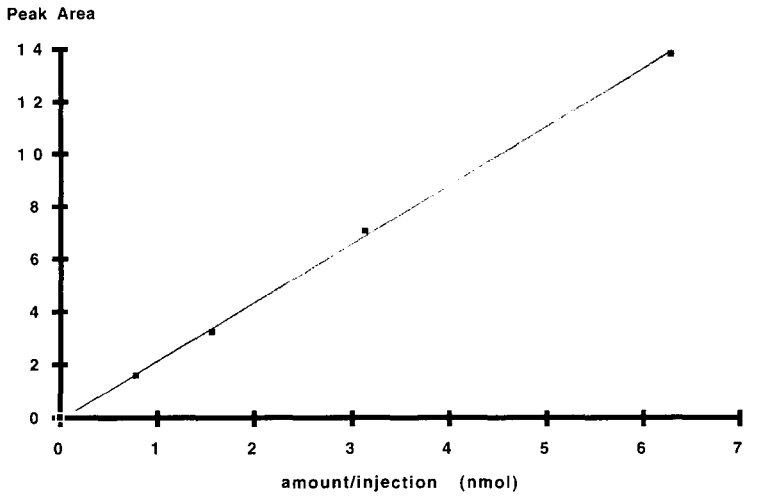


Fig. 6. Standard curve for TBA–β-formyl pyruvic acid, measured as peak area centred at 549 nm, versus the amount of the starting material, ammonium 3-deoxy-2-keto-octonate, used in the analysis.

For quantification purposes, a standard calibration curve was prepared by measuring the HPLC peak area of the TBA–β-formyl pyruvic acid. A linear relationship was obtained in the range of 0–6.3 nmol/injection, as shown in Fig. 6. The lowest detectable amount of TBA–β-formyl pyruvic acid using HPLC separation was found to be about 0.8 nmol/injection.

Under conditions where TBA–β-formyl pyruvic acid is formed, with no or very little concomitant formation of the degradation product having an absorbance maximum at 530 nm, i.e. at 50 °C and around 170 min reaction time, a direct light-absorption measurement at 549 nm can be used. The results obtained are not as exact as the combination with HPLC separation but they are still acceptable. A higher reaction temperature and/or a longer reaction time, however, gives inferior results.

Application to pulps.—Both hardwood and softwood contain xylan as a hemicellulose constituent, although the amount is much higher in a hardwood like birch than in a softwood. The chemical compositions of hardwood and softwood hemicelluloses are different but they both contain 4-*O*-methyl-glucuronic acid moieties. On alkaline treatment, as in kraft pulping, a substantial amount of the xylan is lost by degradation and dissolution. In the remainder, the 4-*O*-methyl-glucuronic acid units are to a considerable degree converted into hexeneuronic acid units [2]. A number of industrial and laboratory-made kraft pulps were purified from extractives by acetone extraction and subjected to the analytical procedure described above in order to further elucidate the presence of hexeneuronic acids in different pulps. The results are presented in Table 1. For each pulp, the kappa number, i.e. the consumption of potassium permanganate, after extraction was measured, according to the standard method SCAN-C 1:77, as a way of determining the total amount of oxidizable material present.

Usually, the kappa number is thought to reflect the amount of residual lignin present in a chemical pulp. However, as shown in Table 1, the contribution from hexeneuronic

Table 1
Hexeneuronic acid found in different pulps and its contribution to the kappa number

Pulp	Kappa number	Hexeneuronic acid, μMol/g pulp	Kappa number equivalence [a]
Birch, unbleached			
	10.3	37	3.2
	11.3	42	3.6
	14.0	64	5.5
	14.5	57	4.9
	15.9	65	5.6
	18.8	67	5.8
Birch, bleached [b]			
	6.0	53	4.6
	4.5	39	3.4
Pine, unbleached			
	18.2	14	1.2
	18.6	22	1.9
	18.0	24	2.1

[a] The relationship between kappa number and amount of hexeneuronic acid will be published elsewhere.
[b] The birch pulp was bleached with oxygen and hydrogen peroxide in alkaline media according to a OQP-sequence.

acid to the kappa number measurement (''kappa number equivalence'') was found to be considerable in all the birch pulps analysed. In pine pulps having a smaller amount of xylan in the fibres, the contribution was smaller. From the limited amount of data from pulps, no firm conclusions can be drawn regarding the influence of pulping parameters on the formation of hexeneuronic acid moieties. A tendency towards lower values at lower kappa numbers is evident, but here additional analytical data are required. The bleached pulp, on the other hand, contained a high residual amount of hexeneuronic acid, and this particular bleaching sequence, although producing a colourless fibre with very little lignin, did not apparently affect the hexeneuronic acid structure.

4. Conclusions

The results obtained in this work demonstrate that hexeneuronic acid groups in chemical pulps can be accurately determined using a combination of selective hydrolysis, periodate oxidation, and coupling with thiobarbituric acid to form a coloured structure which can be quantified by its absorbance at 549 nm. The method is simple and sensitive and it requires only a very small amount of fibres, 10 mg or less. The time required for a complete analysis is of the order of 4 h.

Applied to kraft pulp fibres, the method shows that both birch and pine pulps contain hexeneuronic acid moieties and that, in birch pulps, the contribution to the kappa number is substantial. After bleaching with alkaline oxygen and hydrogen peroxide, the resulting pulp fibres still contain this structure.

Acknowledgements

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