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Determination of reducing end sugar residues in oligo- and polysaccharides by gas–liquid chromatography

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Abstract

Reducing end sugar residues in maltodextrins and arabinoxylans are determined as alditol acetates by gas–liquid chromatography following reduction, acid hydrolysis and acetylation of the samples. After this conversion to alditol acetates, the reducing end sugars are thus separated from their acetylated aldose counterparts. The method allows to identify individual reducing end sugars quantitatively and is a good alternative for colorimetric reducing sugar assays and ^{1}H-NMR analysis. To demonstrate the advantages of the method, an application in a study of enzymic solubilisation and degradation of water unextractable arabinoxylan from a flour squeegee fraction is described.

Keywords: Arabinoxylans; Endoxylanases; Carbohydrates

1. Introduction

Reducing sugar measurement is important in the characterisation of the physicochemical properties of oligo- or polysaccharides. Accurate quantification of the relative amounts of reducing sugars allows one to calculate number average molecular mass and polydispersity indices (in combination with mass average molecular mass) and to predict the reducing power of the components characterised.

Several methods for determination of the reducing sugar content of carbohydrates are available. Apart from the well known dinitrosalicylic acid (DNS) approach [1], the method developed by Somogyi [2] and an adaptation thereof [3] have found widespread use. High-performance size-exclusion chromatography (HPSEC) and high-performance anion-exchange chromatography (HPAEC), within certain limits, provide information on the degree of polymerisation and thus the proportion of reducing end monosaccharide moieties. Proton nuclear magnetic resonance (^{1}H-NMR) [4] has also been used for reducing sugar analysis.

One of the main disadvantages of most of these methods is that they do not allow to determine the identity of the reducing sugar residues. In a mixture, different sugars can often not be identified. As a result, the degradation of molecules with release of distinct reducing sugar residues cannot be adequately described. A second disadvantage is that they are prone to interference from other substances present. ^{1}H-NMR [4] does not have these drawbacks, but is not readily accessible to common use.

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Dutton et al. [5] and Yamaguchi and Okamoto [6] solved these problems by reverting to GLC as an analysis tool for determining reducing end sugar residues. Although the technique of Yamaguchi and Okamoto [6] was successful, it was laborious and time consuming, as it necessitated pre-isolation of the reducing sugar moieties and several sample cleaning steps prior to GLC analysis. Dutton et al. [5] presented a more streamlined procedure where model mixtures of aldoses and alditols were converted to trimethylsilyl derivatives and analysed on a packed GLC column.

The advance in chromatographic techniques has allowed us to develop a method that is much faster than that by Yamaguchi and Okamoto [6] and more sensitive than that by Dutton et al. [5]. It is closely related to a standard technique by Englyst and Cummings [7] as modified by Loosveld et al. [8] for determination of constituent monosaccharides by GLC following hydrolysis, reduction and acetylation of sugar oligomers or polymers (Fig. 1A).

In this paper, the new method is described and tested on mono-, di-, and trisaccharides as well as on characterised maltodextrins. The latter are starch hydrolysates consisting of α-D-glucose units linked by (1→4) glycosidic linkages as well as by (1→6) linkages [9]. To demonstrate the advantages of the technique, enzymic solubilisation of water unextractable arabinoxylan from a wheat flour fraction is given as an example. This polydisperse non starch polysaccharide consists of a β-(1→4) linked D-xylopyranose backbone with no, one or two L-arabinofuranose moieties α-(1→3) and/or α-(1→2) linked to the D-xylopyranose residues. Arabinoxylan levels in wheat flour are in the range of 1.5 to 2.5% of which approximately one third is water extractable and two third water unextractable through physical entrapment or covalent crosslinks [10].

2. Materials and methods

2.1. Chemicals

Bacillus subtilis endoxylanase (EC 3.2.1.8), free from α- and β-amylase, β-glucanase and protease side activities, was obtained from Puratos NV (Groot-Bijgaarden, Belgium). One unit of this enzyme preparation is defined as the volume of enzyme solution needed (μl) to yield a change in extinction of 1.0 at pH 6.0 in the azurine crosslinked arabinoxylan procedure described in Megazyme Data Sheet 8/94 (Megazyme, Bray, Ireland). Xylotriose was from Megazyme. All other reagents were of at least analytical grade and supplied by Sigma-Aldrich (Bornem, Belgium) unless specified otherwise. Standard P-82 pullullans were purchased from Showa Denko (Tokyo, Japan).

2.2. Preparation of maltodextrin fractions

Maltodextrin samples were as previously described by Defloor et al. [11]. They were obtained from commercially available potato starch derived maltodextrins (Paselli MD6, Avebe, Antwerp, Belgium) and waxy maize starch derived maltodextrins (StarDri 5, Amylum, Aalst, Belgium) by fractionation through ethanol precipitation. Maltodextrin sample names are constructed according to the method of isolation [11]. The name P/50/300/Paselli MD 6 e.g., indicates that the sample was obtained as the precipitate in a 50% ethanol solution from 300 g Paselli MD 6 starting material.

2.3. Solubilisation of water unextractable arabinoxylan in a flour squeegee fraction by use of an endoxylanase

The flour squeegee fraction was obtained though fractionation of the flour in its four main components, i.e. gluten, prime starch, a water soluble fraction and the squeegee fraction, by dough formation, washing and centrifugation and, finally, freeze drying. It consists mainly of the small or damaged starch granules, bran particles and the water unextractable arabinoxylan [12].

Squeegee fractions (1.50 g) were suspended in 10.0 ml NaCl solution (0.3%, w/v) containing 0.06 Units of *B. subtilis* endoxylanase. They were incubated at 30°C under continuous shaking. After 15, 90, 240 or 480 min incubations, they were centrifuged (9000 *g*, 10 min, 15°C). The supernatants were boiled for 15 min to destroy enzyme activity. The residues were washed with water (10.0 ml) and centrifuged as above. The obtained wash waters were combined with the corresponding supernatants. After

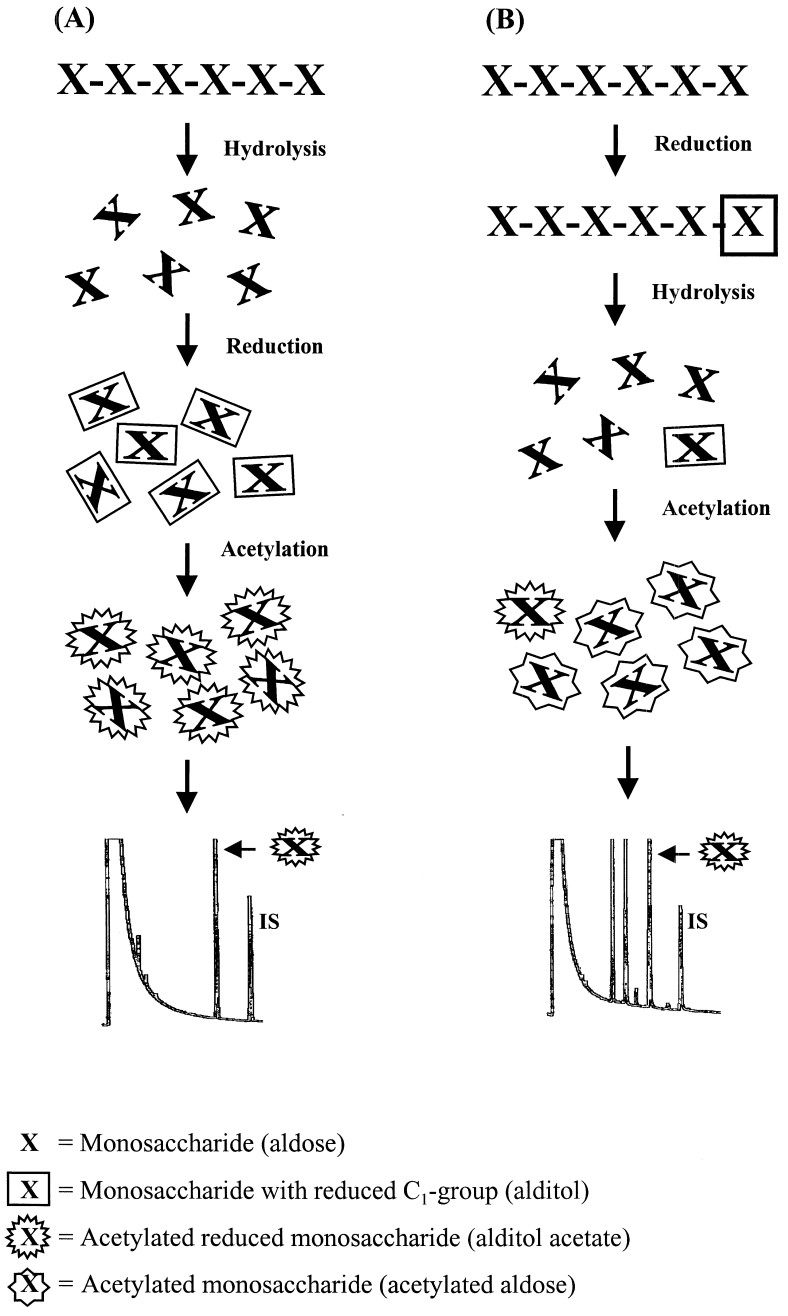

Fig. 1. Schematic representation of (A) the procedure followed for total sugar analysis of a polysaccharide containing sample by Loosveld et al. [8] and (B) the new procedure followed for analysis of the reducing end sugar moieties of a polysaccharide containing sample.

a second centrifugation step as above, the supernatants were analysed by HPSEC and GLC, both as outlined below. A control sample, which lacked the enzyme, was incubated for 30 min at 30°C and received the same treatment as the other samples. It was included to determine whether residual water

extractable arabinoxylan or endogenous amylases were present in the squeegee fraction.

2.4. Analysis of monosaccharide composition after hydrolysis

Monosaccharide composition after hydrolysis of the maltodextrins and the arabinoxylan containing supernatants was estimated by GLC as described by Loosveld et al. [8]. Hydrolysis was thus with 2.0 *M* trifluoroacetic acid (TFA), reduction with $NaBH_4$ and acetylation with acetic acid anhydride. Arabinoxylan content was then defined as 0.88 times the sum of the monosaccharide xyloses and arabinoses (corrected for the presence of arabinogalactan as outlined by Loosveld et al. [8]). Polymeric glucose content was defined as 0.9 times the monosaccharide glucose content.

2.5. Analysis of reducing end sugar content

The reducing end sugar composition of the maltodextrins and supernatants was estimated by GLC. Depending on the expected reducing end sugar content, maltodextrins (10.0–100.0 mg) were solubilised in 2.5 ml water in small screwcap test tubes. D-allose (0.5 ml of a 100.0 mg in 100 ml 50% saturated benzoic acid solution) was added as internal standard, followed by NH_3 (25%, 50 μl) and several droplets of octanol. After 30 min incubation with $NaBH_4$ (0.2 ml; 200 mg in 1 ml NH_3 2.0 *M*) at 40°C and with closed caps, 400 μl acetic acid was added very slowly as foaming tended to occur. Aliquots (2.5 ml) were transferred to a second small screwcap test tube. Concentrated TFA (0.5 ml) was added, resulting in a 2.0 *M* TFA solution. The samples were hydrolysed with closed screwcaps for 1 h at 110°C. After transferring 500 μl of the hydrolysed samples in a large screwcap test tube, 0.5 ml 1-methyl imidazole and 5.0 ml of acetic anhydride were added. The solutions were vortexed and allowed to rest for 10 min. Ethanol (0.9 ml) was added. After 5 min, water (10.0 ml) was added and samples were again vortexed. Bromophenolblue (0.5 ml; 0.04 g in 100 ml water) was added and the test tubes were placed in ice water. Potassium hydroxide solution (7.5 *M*) was added (2×5.0 ml with an intermediate rest of 5 min). Test tubes were mixed one last time. Following phase separation, the upper, organic phase was removed by a pasteur pipette and dried with anhydrous Na_2SO_4. It was then transferred to vials. Glass inserts were used to avoid dilution.

Samples (1.0 μl) were separated on a Supelco SP-2380 polar column (30 m×0.32 mm I.D., 0.2 μm film thickness) (Supelco, Bellefonte, PA, USA) in a Chrompack 9011 chromatograph (Chrompack, Middelburg, The Netherlands) equipped with autosampler, splitter injection port (split ratio 1:100) and flame ionizing detector. The carrier gas was He (column head pressure 63 kPa). Separation was at 210°C, with injection and detection at 275°C.

In this procedure reducing end aldoses, e.g., are first reduced to alditols. Following hydrolysis, they are acetylated and thus result in alditol acetates. The non-reducing aldoses in a polymer chain cannot be reduced. Following hydrolysis, they are transformed into acetylated aldoses that are still subject to conformational changes through mutarotation. The resulting chromatograms thus contain several peaks from each of the acetylated aldoses and one peak from each of the alditol acetates. Identification and quantification of the latter is through analysis in the procedure of calibration mixtures containing only the monomeric form of the reducing end sugars under study and the internal standard (IS). A schematic overview of the procedure is presented in Fig. 1.

2.6. High-performance size-exclusion chromatography

Supernatants (20 μl) obtained after enzymatic treatment of the flour squeegee fraction were separated on a Shodex SB-806 HQ GPC column (300 mm×8 mm I.D.) with a Shodex SB-G guard column (50 mm×6 mm I.D.) from Showa Denko. Elution was with 0.3% NaCl (0.5 ml min^{-1} at 30°C) on a Kontron 325 pump system (Kontron, Milan, Italy) with autoinjection. The separation was monitored with a refractive index detector (VSD Optilab, Berlin, Germany). Molecular mass markers were Shodex standard P-82 pullulans (1.0 mg ml^{-1}) with molecular masses of $78.8 \cdot 10^4$, $40.4 \cdot 10^4$, $21.2 \cdot 10^4$, $11.2 \cdot 10^4$, $4.73 \cdot 10^4$, $2.28 \cdot 10^4$, $1.18 \cdot 10^4$ and $0.59 \cdot 10^4$ and glucose.

3. Results and discussion

3.1. Reducing sugar residue contents determined by the combined dubois hizukuri^{-1} method, ^{1}H-NMR and GLC

To validate the new method described here, monomeric xylose, maltose, xylotriose and maltodextrin samples were analysed for both total sugar content and reducing sugar residue contents by GLC. For maltodextrins, the results were compared with those obtained by the combination of the phenol sulfuric acid method for total sugar content [13] and the Hizukuri method for reducing sugar content through reduction of ferri-ions [3] and by ^{1}H-NMR analysis [4] as described by Defloor et al. [11]. It is clear from Table 1 that the GLC method is quite accurate. For the maltodextrin samples, it tends to yield somewhat lower reducing sugar values than the combined colorimetric methods and, to a lesser extent, ^{1}H-NMR analysis. For the former, this difference can possibly be ascribed to the non selective nature of both colorimetric methods for sugar determination. Although little contamination was found in the maltodextrin samples, small impurities with reducing properties are likely to influence reducing sugar residue contents determined this way. This probably also explains the differences found between the combined colorimetric method and ^{1}H-NMR estimates, especially for the StarDri 5 derived maltodextrins.

The new GLC method is also reproducible. A maximum deviation of 4.5% was found with at least five measurements per sample spread over several days.

3.2. Arabinoxylan and starch solubilisation in a flour squeegee fraction

The HPSEC profiles of the supernatants at different incubation times with the endoxylanase are given in Fig. 2. The increase in curve area with incubation time points to increasing solubilisation with time as confirmed by analysis of the total sugar content and composition of the supernatants after hydrolysis (Table 2). More than 80% of the arabinoxylan in the squeegee fraction was solubilised after 8 h incubation. The arabinose over xylose ratio of the solubilised arabinoxylan, an important structural characteristic, did not change significantly during this period. Solubilisation was accompanied by a substantial release of glucose compounds from the residue. Even more glucose (6.40% of the original

Table 1
Comparison of the average degree of polymerisation (DP) of xylose, maltose, xylotriose and maltodextrin samples as measured by the combined Dubois Hizukuri^{-1} methods, ^{1}H-NMR and GLC-analysis

Sample[a]	Colorimetric[a]	^{1}H-NMR[a]	GLC[b]
Xylose	–	–	0.98 (0.03)[c]
Maltose	–	–	2.07 (0.08)
Xylotriose	–	–	3.02 (0.09)
P/50/300/StarDri 5	61	68	72.7 (1.3)
P/75/300/StarDri 5	19	21	22.0 (0.3)
SN/300/StarDri 5	4	5	4.4 (0.1)
P/50/300/Paselli MD6	66	66	74.8 (2.0)
P/75/300/Paselli MD6	17	18	17.6 (0.3)
SN/300/Paselli MD 6	5	5	5.7 (0.2)
P/50/1/Paselli MD 6	251	–	275.3 (9.2)
P/75/1/Paselli MD 6	42	–	39.0 (1.1)
SN/1/Paselli MD 6	7	–	7.7 (0.3)

[a] Maltodextrin samples as described by Defloor et al. [11].
[b] $DP=(\%MS_{Xyl\ or\ Glu} \div \%RS_{Xyl\ or\ Glu})$, with MS: monosaccharide, RS: reducing end sugar, Xyl: xylose and Glu: glucose.
[c] Values between brackets are standard deviations.

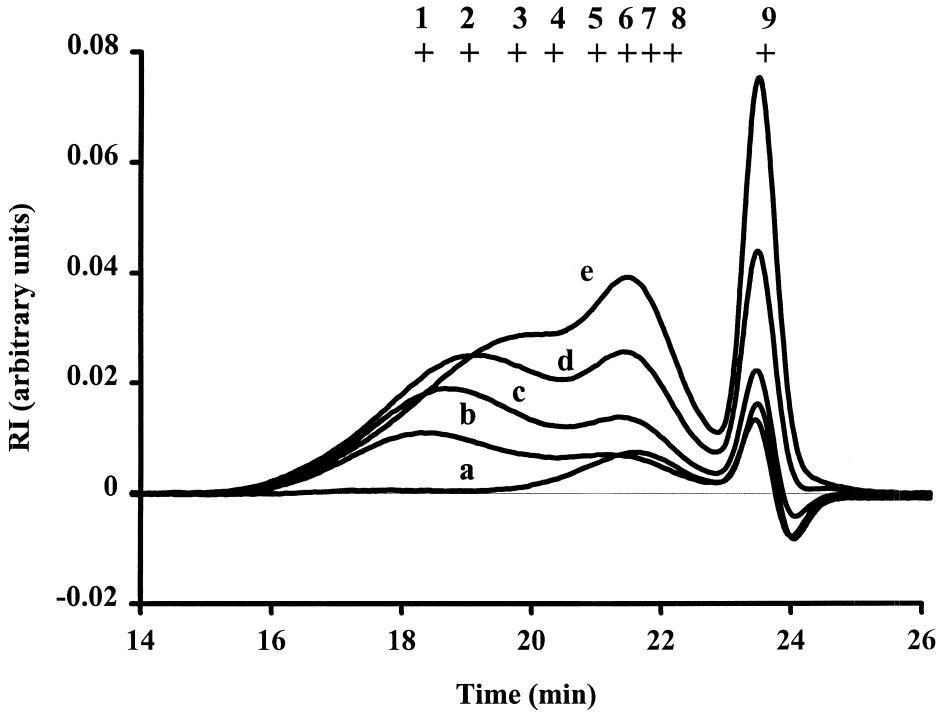

Fig. 2. HPSEC profiles of the supernatant after incubation of a flour squeegee fraction with endoxylanase in water for (a) 0 min (control), (b) 15 min, (c) 90 min, (d) 240 min and (e) 480 min. Pullullan calibration standards are 78.8×10^4, 40.4×10^4, 21.2×10^4, 11.2×10^4, 4.73×10^4, 2.28×10^4, 1.18×10^4, 0.59×10^4 and glucose, numbers **1** through **9** respectively.

sample) than arabinoxylan (5.43%) was released at the longest incubation time.

As the supernatants did not only contain arabinoxylan but also glucose in considerable amounts, the HPSEC profiles did not allow to make conclusions on the molecular mass of the arabinoxylan present. Reducing end sugar residue analysis by the new GLC method allowed further characterisation because different reducing end sugar residues could be separated. Combined with total sugar analysis, the

Table 2
Original composition of the squeegee fraction (% dry matter), arabinoxylan (AX) and glucose solubilisation (% of starting material dm), with corresponding arabinose over xylose ratio (A/X), average degree of polymerisation (DP_{AX} and DP_{Glu}) upon increasing incubation time of the squeegee fraction with endoxylanase

Incubation time (min)	AX[a]	A/X	DP_{AX}[b]	Glu[c]	DP_{Glu}[d]
Squeegee fraction	6.19	0.56	–	91.3	
Control	0.20	0.53	65 (3)	1.61	4.2 (0.1)
15	2.19	0.49	498 (37)	1.23	4.0 (0.2)
90	3.79	0.49	602 (28)	2.26	4.1 (0.1)
240	5.04	0.50	488 (23)	4.26	3.9 (0.1)
480	5.43	0.51	314 (6)	6.40	3.4 (0.1)

[a] AX=[(% MS_{Ara}+%MS_{Xyl})×0.88]. Standard deviation<4.0%.
[b] DP_{AX}=[(% MS_{Ara}+% MS_{Xyl})÷% RS_{Xyl}]. Values between brackets are the standard deviations.
[c] Glu=[% MS_{Glu}×0.9]. Standard deviation<4.0%.
[d] DP_{Glu}=[% MS_{Glu} ÷% RS_{Glu}]. Values between brackets are the standard deviations.

average degree of polymerisation (DP) of arabinoxylan and glucose for each sample was calculated. Results are summarized in Table 2.

Glucose appeared to be present as monomers and oligomers, while arabinoxylans were present as polymers with large DP and a large number average molecular mass accordingly. Thus, because the low molecular mass peak of the HPSEC profiles could be largely, if not completely, attributed to glucose components, we omitted this peak for calculation of weight average molecular mass estimates from the HPSEC profiles [12]. The results are presented in Table 3 together with the peak average molecular mass.

Taken together, the HPSEC profiles in Fig. 2 and the physicochemical characteristics of the supernatants in Tables 2 and 3 indicate that enzymic breakdown first results mainly in the release of large arabinoxylans from the squeegee fraction. With time, degradation of solubilised material to smaller arabinoxylan fragments becomes prevalent. Both actions occur simultaneously to some degree during incubation and a very polydisperse hydrolysate is obtained at all times.

No reducing end arabinose residues were detectable at any given time. This indicates that no monomeric arabinose was released and thus no arabinofuranosidase side activity was present in the endoxylanase preparation used.

Glucose oligomers with very small DP are released continuously in the supernatant. As the *Bacillus subtilis* endoxylanase was free of α- and β-amylase activity, this is due to residual amylase activity in the squeegee fraction itself. This was confirmed by the results for the control sample. Indeed, in the control sample, which was incubated for 30 min without endoxylanase added, more glucose was solubilised compared to the sample incubated with the endoxylanase for 15 min.

The error on three replicate measurements was rather low. A standard deviation lower than 5.0% was found for both the reducing sugar and the degree of polymerisation data, except for one sample where a deviation of 7.5% was found.

The lower limit for detection of reducing sugar residues in a sample was 5 μg. Through partial evaporation of the organic phase obtained at the end of sample preparation this sensitivity was increased to 1 μg. Submicrogram sensitivity can be obtained by decreasing the split ratio of the gas chromatograph injector.

Table 3
Weight-average (M_w), peak (M_p) and number-average (M_n) molecular mass estimates of arabinoxylans solubilised by endoxylanase treatment of a flour squeegee fraction

Incubation time (min)	M_w [a]	M_p	M_n [b]
Control	27 000	16 000	9000
15	729 000	741 000	66 000
90	615 000	647 000	79 000
240	530 000	374 000	64 000
480	383 000	22 000	42 000

[a] M_w estimates calculated from HPSEC results with exclusion of the low molecular mass peak.

[b] M_n calculated from reducing end sugar analysis by GLC.

4. Conclusion

The method described here for analysis of reducing end sugar residues by GLC correlates well with other methods. Its main advantage is that it analytically distinguishes different reducing sugar residues. This enables us to better characterise the reducing moieties of samples under study and is of interest for widely varying applications ranging from Maillard reactions to structural analysis. An example of the latter clearly demonstrated this feature. The similarity of this method to a method for determination of total sugar content and composition through GLC is convenient both practically and technically.

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