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Bilberry xyloglucan—novel building blocks containing β-xylose within a complex structure

Hauke Hilz, Laura E. de Jong, Mirjam A. Kabel, René Verhoef, Henk A. Schols and Alphons G. J. Voragen*

Wageningen University, Department of Agrotechnology and Food Sciences, Laboratory of Food Chemistry, PO Box 8129, 6700 EV Wageningen, The Netherlands

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Abstract—Bilberries are known to have one of the most complex xyloglucan structures described in the plant kingdom until now. To characterise this structure, xyloglucans were enzymatically degraded and the oligosaccharides obtained were analysed. More than 20 different building blocks were found to make up the xyloglucan polymer. Bilberry xyloglucan was of XXXG-type, but some XXG-type oligomers were present, as well. The building blocks contain galactose–xylose (L) and fucose–galactose–xylose (F) side chains. In both side chains, the galactose units can be acetylated. In addition, β-xylose-α-xylose (U) side chains were shown. This U chain was present in three building blocks described before (XUXG, XLUG and XUFG) and four novel blocks that have not been described in the literature previously: XUG, XUUG, XLUG and XXUG.

Keywords: *Vaccinium myrtillus*; Bilberry; Cell wall polysaccharides; Xyloglucan; Hemicellulose; β-Xylose; Ericales; Berries; Capillary electrophoresis

1. Introduction

Xyloglucans are the main hemicelluloses in cell walls of dicotyledons,[1,2] where they were first described in sycamore cells.[3]

As an interlink between cellulose microfibrils, xyloglucans are assumed to be part of the skeletal framework as depicted in several models of the plant cell wall.[4,5] Within the cellulose–xyloglucan network three xyloglucan domains are described: the first domain includes the parts of xyloglucans that bridge the space between cellulose microfibrils or that form free loops. This domain can be degraded by *endo*-glucanases. The major part of xyloglucans belongs to the second domain that covers the cellulose microfibrils and can be extracted with concentrated alkali. The third domain of the xyloglucans is entrapped within the amorphous cellulose microfibrils, which have to be degraded before the xyloglucan is accessible for enzymatic degradation or extraction.[5–7]

A β-(1→4)-linked glucan chain forms the backbone of xyloglucans.[8] Different short side chains are attached in position 6 to this backbone.[3] These chains were assigned a one-letter code to simplify nomenclature.[9] Some examples for the side chains are G for an unsubstituted β-D-Glc*p*, X for an α-D-Xyl*p*-(1→6)-β-D-Glc*p* unit, L for a β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)-β-D-Glc*p* unit, F for a α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)-β-D-Glc*p* unit, or S for an α-L-Ara*f*-(1→2)-α-D-Xyl*p*-(1→6)-β-D-Glc unit.

Xyloglucans can be degraded by *endo*-glucanase in a regular way. Oligomers formed always contain an unbranched glucose at the reducing end. The polymeric xyloglucans are classified by the types of oligomers formed after enzymatic degradation. Xyloglucan

Abbreviations: AIS, alcohol insoluble solids; APTS, 8-aminopyrene-1,3,6-trisulfonate; CASS, concentrated alkali soluble solids; CE, capillary electrophoresis; ESIMS, electrospray ionisation mass spectrometry; HPAEC, high performance anion exchange chromatography; LIFD, laser-induced fluorescence detection; MALDI-TOFMS, matrix assisted laser desorption time of flight mass spectrometry; PAD, pulse amperometric detection; SPE, solid phase extraction; XEG, xyloglucan specific *endo*-glucanase.

* Corresponding author. Tel.: +31 317 482888; fax: +31 317 484893; e-mail: fons.voragen@wur.nl

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oligomers of XXXG-type, XXGG-type, or XXGGG-type structure are the most common patterns.[10,11] XXXX-type xyloglucan of which every glucosyl residue in the backbone is substituted with arabinosyl residues in position 2 was also reported.[12]

The structure of xyloglucans is regular and similar within one botanic family.[1] Bilberries belong to the family of Ericaceae, order Ericales. The xyloglucan structure of one other member of this order has been described before: the argan tree (*Argania spinosa* (L.) Skeels), which belongs to the family of Sapotaceae.[13] In these xyloglucans, a side chain of β-D-Xyl*p*-(1→2)-α-D-Xyl*p*-(1→6)-β-D-Glc*p* was identified for the first time and encoded with U. The building block XUFG was identified by NMR analysis. Based on MS data only, XUXG and XULG were proposed to be present, as well.

Bilberries (*Vaccinium myrtillus* L.) are an important crop in northern Europe due to their unique taste and flavour, although they are not domesticated. The berries are mainly used for juice production. We characterised cell wall polysaccharides of bilberries recently and showed high amounts of xylose and glucose in their hemicellulose rich fraction, indicating the presence of xyloglucan.[14,15]

The aim of this study was to analyse xyloglucan from bilberries. To show the presence of xyloglucans and to characterise their structure, xyloglucans were degraded by xyloglucan specific *endo*-glucanase (XEG)[16] and the obtained oligomers were analysed using HPAEC, CE and mass spectrometry.

2. Results

Xyloglucans of bilberries were analysed following two approaches. In a first approach, hemicelluloses were extracted with 6 M sodium hydroxide from residual cell wall material after removal of pectins (concentrated alkali soluble solids; CASS). Xyloglucans were further purified on an anion exchange column, where neutral hemicelluloses including xyloglucan were separated from anionic hemicelluloses and the remaining pectins. Under these alkali extraction conditions, the possible acetyl esters were saponified and could not be determined. Therefore, xyloglucans were degraded with XEG directly starting from alcohol insoluble solids (AIS) in a second approach. During this procedure the possible acetyl esters remained intact on the obtained oligosaccharides.

2.1. Sugar composition of xyloglucan containing fractions

For a first characterisation of polysaccharides in CASS and AIS, the sugar composition after hydrolysis of the polysaccharides was determined (Table 1). In CASS, xylose and glucose were the major sugar residues with a ratio of xylose to glucose typical for xyloglucan (3:4). Only small amounts of uronic acids were present in this fraction in a ratio 5:1 GalA to GlcA.[15]

Purer xyloglucan was obtained when CASS was further fractionated on an anion exchange column.[17] Eighteen percent of CASS did not bind to the column and eluted with 5 mM sodium acetate buffer (CASS unbound). This fraction contained partly purified xyloglucan as indicated by its sugar composition (Table 1). CASS unbound contained xylose and glucose in a 1:2 ratio. The amount of fucose was relatively high in this fraction and arabinose was hardly present. The amount of uronic acids was low. Probably xyloglucans with fucose and galactose containing side chains (galactose content 13 mol %) were present and arabinose was not part of bilberry xyloglucans.

The major sugar residues in bilberry AIS were xylose, glucose, and uronic acid. The molar ratio of xylose to glucose was almost 1:1 showing that not only xyloglucan, but also xylans were present in the cell wall material of bilberries. Bilberry seeds contain high amounts of xylans.[15]

2.2. Profiling of xyloglucan oligomers by MALDI-TOFMS and ESIMS^n

Endoglucanase II is a xyloglucan specific *endo*-glucanase (XEG), which is known to split xyloglucan between an unbranched glucose and a xylose-substituted glucose residue.[16] By incubation with XEG, xyloglucans are degraded into specific oligosaccharides representing the building blocks of xyloglucans,[9] which can have XXXG type, XXGG type, or the XXGGG type structure.[10,11]

MALDI-TOFMS of XEG digested xyloglucans indicates the presence of different oligomers by their mass to charge ratios. When ESIMS^n is performed, the structure of the oligomers can be identified. However, the different hexoses and pentoses cannot be distinguished. By comparing xyloglucan oligomers obtained from CASS with the ones obtained from AIS, acetylation profiles can be seen.[1,2]

The MALDI-TOF mass spectrum of xyloglucan oligomers derived from bilberry CASS is shown in Figure 1a.

Table 1. Sugar composition of xyloglucan containing fractions of bilberries (mol %)

	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	Total sugars (w/w%)	Yield
CASS	1	1	3	28	11	12	38	6	66	4% of AIS
CASS unbound	1	4	1	19	16	13	42	6	39	18% of CASS
AIS	1	0	5	29	2	5	35	22	40	4% of fresh berries

In this spectrum 19 peaks were present. Fifteen peaks were attributed to sodium adducts of xyloglucan oligosaccharides and four peaks were attributed to potassium adducts (1233, 1263, 1425, 1571), which have a mass to charge ratio of 16 higher than their corresponding sodium adducts. The xyloglucan oligomers XXXG (1085), XXLG and XLXG (X[XL]G; 1247), XXFG (1393), XLLG (1409), and XLFG (1555) were identified according to their mass to charge ratio[1] and their ESIMS^2 spectra. Other major peaks were present, which were attributed to oligomers that contained either a side chain of two pentoses or two pentoses attached to one glucose residue of the backbone. Until further identification, this side chain is coded as (1H+2P). The main xyloglucan oligomers containing these side chains were X[X(1H+2P)]G (1217), X(1H+2P)(1H+2P)G (1349), X[L((1H+2P)]G (1379), and X(1H+2P)FG (1525). Some smaller peaks present were attributed to X(1H+2P)G (923), XLG (953), and XLGG (1115). The ESIMS^2 spectrum of the peak with the mass to charge ratio of 1085 suggested the presence of small amounts of X(1H+2P)GG next to XXXG. Three oligomers with the mass to charge ratios of 1277, 1511 and 1541, respectively, were shown to carry a substituted xylose residue at the nonreducing end. We propose L[LG]G, L(1H+2P)(1H+2P)G and L(1H+2P)LG as most probable structures based on ESIMS^2 analysis, because ^{1}H NMR analysis did not result in conclusive spectra (data not shown). This would mean that XEG is, under special circumstances, able to release xyloglucan oligosaccharides with an L-unit on the nonreducing end.

2.3. Identification of the sugar residues carrying the acetyl group by ESIMS^n

The MALDI-TOF mass spectrum of xyloglucan oligosaccharides derived from AIS showed many more peaks (Fig. 1b) than the one of enzymatically degraded CASS xyloglucans. The additional peaks were the single or double acetylated oligomers. A peak with the mass to charge ratio of XXG (791) was shown additionally compared to the spectrum of xyloglucan oligosaccharides derived from CASS. Two other additional peaks had the mass to charge ratio of 1055 and 1097, respectively. These peaks were attributed to an oligomer composed of four pentoses and three hexoses in the acetyl free and the single acetylated form. Its structure, however, could not be identified. Two peaks were not attributed to xyloglucan oligosaccharides (*m/z* 1229, 1481).

By ESIMS^n the sugar residues that carry the acetyl group were identified. Only oligomers containing a galactose unit in an L or F chain and oligomers containing another G unit next to the reducing end were shown to be acetylated. ESIMS^n analysis confirmed acetylation of the galactose units and the unsubstituted, nonreducing glucose units. When double acetylation was shown for one molecule, no double acetylated residues, but two different acetylated galactose residues or an acetylated galactose and an acetylated glucose unit were present. Among the acetylated xyloglucan oligomers, one peak with a mass to charge ratio of 1127 (1085 + acetyl) was detected. These oligomers were attributed to the tentative structure of acetylated X(1H+2P)GG. The L[LG]G and L[(1H+2P)L]G oligomers were shown in

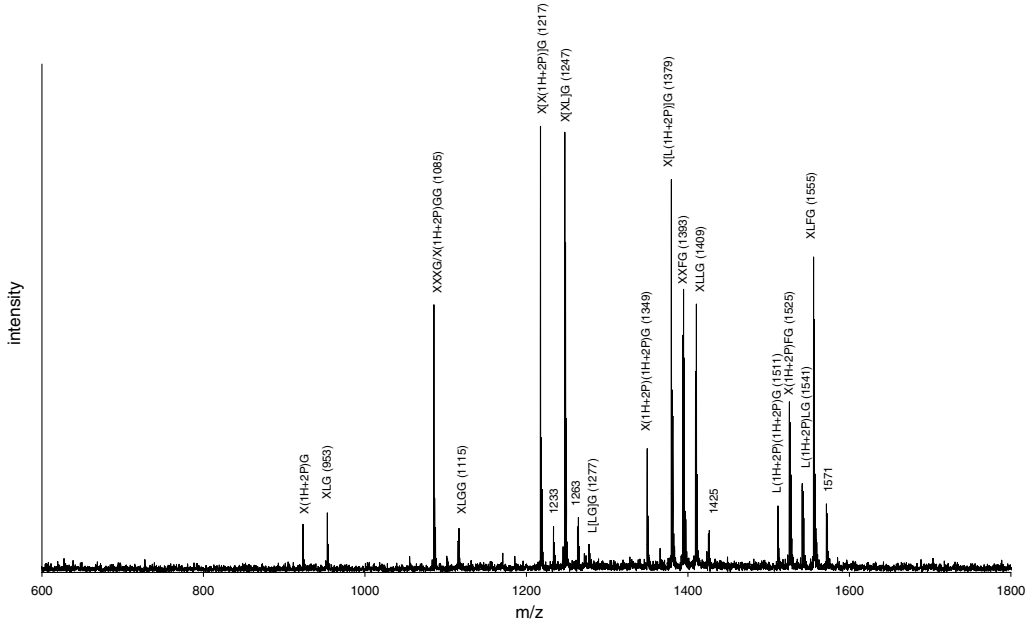

Figure 1a. MALDI-TOF mass spectrum of xyloglucan oligosaccharides derived from bilberry CASS (*m/z* of sodium adducts between brackets). The side chain (1H+2P) was later identified as the U chain by NMR.

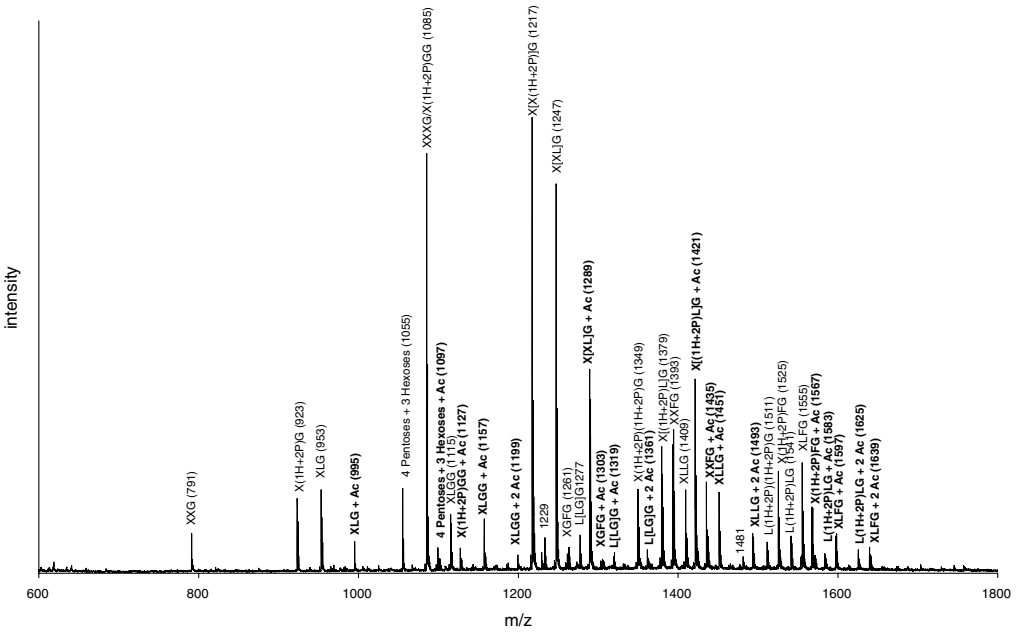

Figure 1b. MALDI-TOF mass spectrum of xyloglucan oligosaccharides derived from bilberry AIS (*m*/*z* of sodium adducts between brackets). The side chain (1H+2P) was later identified as the U chain by NMR spectroscopy.

the MALDI-TOF mass spectrum of xyloglucan derived from AIS, as well, L[LG]G even in the single and double acetylated form.

2.4. Xyloglucan oligosaccharides of bilberries analysed by HPAEC

The HPAEC elution pattern (CarboPac PA 100 column) of xyloglucan oligomers derived from bilberry CASS unbound is shown in Figure 2. The high number of different oligomers made it difficult to separate all oligomers. Bilberry xyloglucans consisted of more than 20 different oligomeric building blocks. For identification, retention times of known xyloglucan oligosaccharides from soy,[18] olive,[2] apple[19] and tamarind[20] were used. Because not all xyloglucan oligomers were identified by this way, off line MALDI-TOFMS and ESIMS^2 were performed after desalting by solid phase extraction[17] in order to identify the still unknown peaks and to confirm the structure of the identified ones.

The smaller oligomers XXG, XFG and XLG eluted first from the HPAEC column. These oligomers with a backbone of three glucose units were identified by their mass to charge ratios (MALDI-TOFMS) and their ESIMS^2 spectra. XFG was not detected by MALDI-TOFMS of the xyloglucan oligomer mixtures derived from CASS or AIS. These XXG-type oligomers were present as building blocks of bilberry xyloglucans in minor quantities. The major building blocks were of XXXG-type. The first xyloglucan oligomer of XXXG-type eluting from the column was XXXG followed by XXFG, XLFG, and XXLG. XLLG was present in smaller amounts. More oligomers carrying the (1H+2P) side chain were identifed by their mass to charge ratios (MALDI-TOFMS) and their ESIMS^2 spectra.

2.5. Identification of the xyloglucan oligosaccharides by HPAEC-off line-ESIMS^2

The ESIMS^2 spectra of the xyloglucan oligomers separated by HPAEC were spectra of single oligomers. Even if two oligomers were coeluting, they did not have the same molecular mass. The ESIMS^2 spectrum of XX(1H+2P)G 1 is shown in Figure 3 as an example for ESIMS^2 analysis. The peaks present were assigned to fragments of the molecule according to Domon and Costello.[21] Three peaks in the spectrum gave important information about the structure of the oligomer. One of them was the peak with the mass to charge ratio of the four glucose units in the backbone (689). This ion was formed by cleavage of four nonreducing pentose residues resulting in the Y-ion of the backbone. The other peaks represented the fragments which were formed by cleavage in the middle of the oligomer. The peak with a mass to charge ratio of 611 could be either the B3α' or the Z2α' ion. Probably a mixture of the ions was present. The peak with a mass to charge ratio of 629 was probably a mixture of the corresponding ions C3α' and Y2α', respectively. These two peaks showed that two xylose units were attached to the glucose unit of the chain next to the reducing end (a). The other oligomers that contained the (1H+2P) side chain were identified as XL(1H+2P)G, X(1H+2P)LG, X(1H+2P)FG, and X(1H+2P)(1H+2P)G. Three oligomers with the

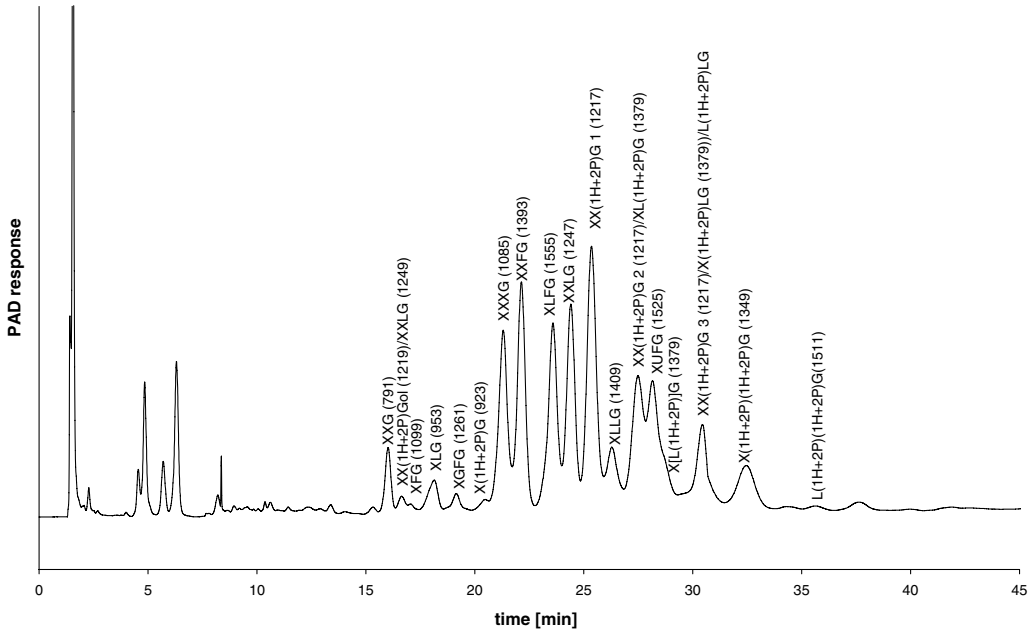

Figure 2. Oligomers obtained by incubation of isolated bilberry xyloglucan (CASS unbound) with XEG on HPAEC CarboPac PA100 column. The side chain (1H+2P) was later identified as the U chain by NMR.

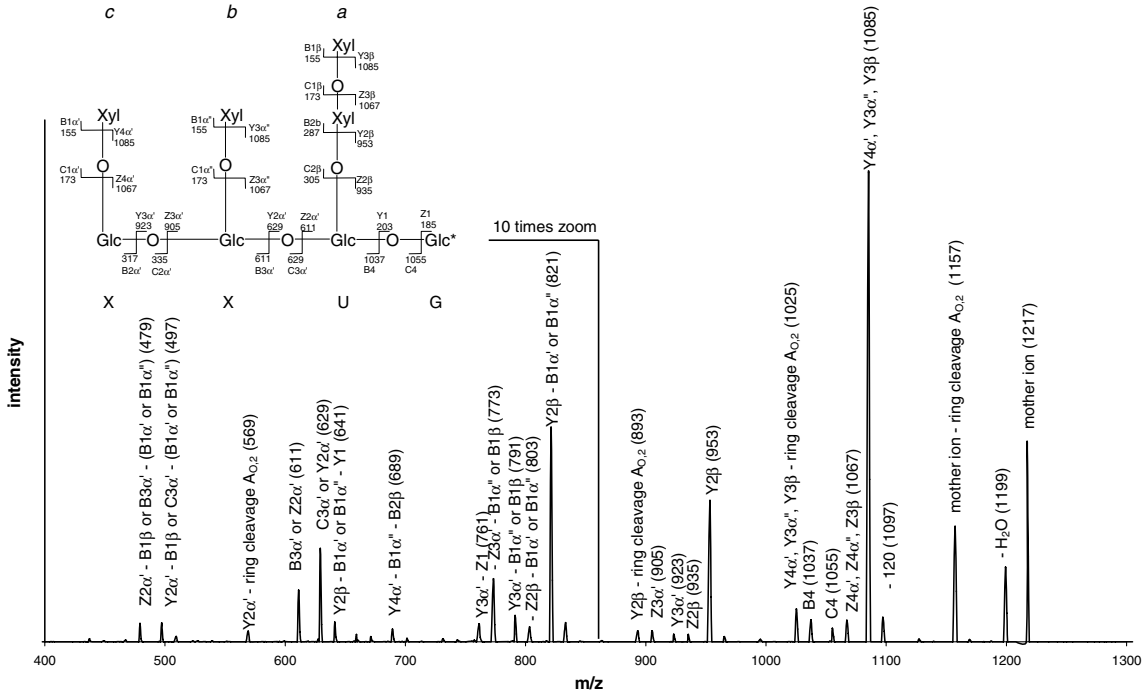

Figure 3. ESIMS^2 spectrum of XX(1H+2P)G (Structure in the upper left corner. (1H+2P) was later identified as U by NMR).

same masses (*m/z* 1217, $[M+Na]^+$) and identical ESIMS^2 spectra were eluted at three different retention times for the HPAEC conditions used. They carried the (1H+2P) side chain next to the reducing end and were labelled XX(1H+2P)G 1–3, although the reason for the different physico-chemical behaviour of the three oligomers on HPAEC cannot be explained. A similar phenomenon was observed for the oligomers carrying a (1H+2P) and an L side chain (*m/z* 1379, $[M+Na]^+$). Next to XL(1H+2P)G and X(1H+2P)LG, a third oligomer eluted from the column at a different retention time. The ESIMS^2 spectrum did not give conclusive spectra. Therefore, the oligomer was labelled X[L(1H+2P)]G. In very small quantities, two oligomers with a mass to

charge ratio of two higher than XX(1H+2P)G or XXLG were shown. According to their ESIMS^2 spectra, they could be the reduced forms XX(1H+2P)Gol and XXLGol, which may be artefacts of the procedure or originally present on the reducing end of xyloglucan.

2.6. CE of bilberry xyloglucan oligosaccharides

In addition to HPAEC, capillary electrophoresis (CE) was used to separate especially acetylated xyloglucan oligosaccharides.[17,22] Xyloglucan oligosaccharides derived from bilberry CASS (Fig. 4a) and AIS (Fig. 4b) were labelled with APTS on the reducing end and the electropherograms were recorded using a laser induced fluorescence detector. Most of the many different oligomers in bilberry xyloglucan were separated within only two minutes. Because LIFD is not able to identify oligomers, CE was also connected online to ESIMS^n. Next to the letter codes, the mass to charge ratios of the triple negatively charged, APTS labelled oligomers are shown in the LIFD-electropherograms.

The electrophoretic mobility of the APTS-labelled oligosaccharides derived from CASS depended on the molecular mass of the oligomers. The oligomers with the lowest molecular mass migrated the fastest to the detector and the ones with the highest molecular mass the slowest. In the beginning of the electropherogram, the resolution was better compared to the end. While XXLG and XLXG with the same mass were separated in different peaks, XL(1H+2P)G, X(1H+2P)LG, and XXFG were detected in one peak. The same major oligomers were detected by CE-LIFD as by HPAEC. It was, however, not possible to identify the oligomers present in the CASS digest in smaller amounts by their masses due to the short separation time and therefore the limited number of mass spectra.

When acetylated oligomers derived from bilberry AIS were analysed, the acetyl free and the monoacetylated forms were not separated from each other. The diacetylated oligomers were, however, separated from the acetyl free and the monoacetylated oligomers, for example, in the case of XLFG and XLLG. In addition, higher amounts of the smaller oligomers were present, so that an identification of the oligomers with an electrophoretic mobility between XXG and XXXG was partly possible. However, some peaks remained unidentified. An oligomer with the mass of three hexoses and four pentoses, which was already shown to be present by MALDI-TOFMS, was attributed to well separated peak. This oligomer was not detected by HPAEC.

2.7. Identification of novel xyloglucan oligosaccharides by ^{1}H NMR spectroscopy

To identify the structure of oligomers containing the unknown (1H+2P) chain, four oligomers (XX(1H+2P)G, X(1H+2P)FG, XL(1H+2P)G, and X(1H+2P)(1H+2P)G) were isolated by preparative HPAEC.[2] The reducing end of the oligomers was reduced to alditols prior to ^{1}H NMR. The chemical shifts and coupling constants (Table 2) were compared to previously recorded spectra of xyloglucan oligosaccharides with[13] and without[23] the

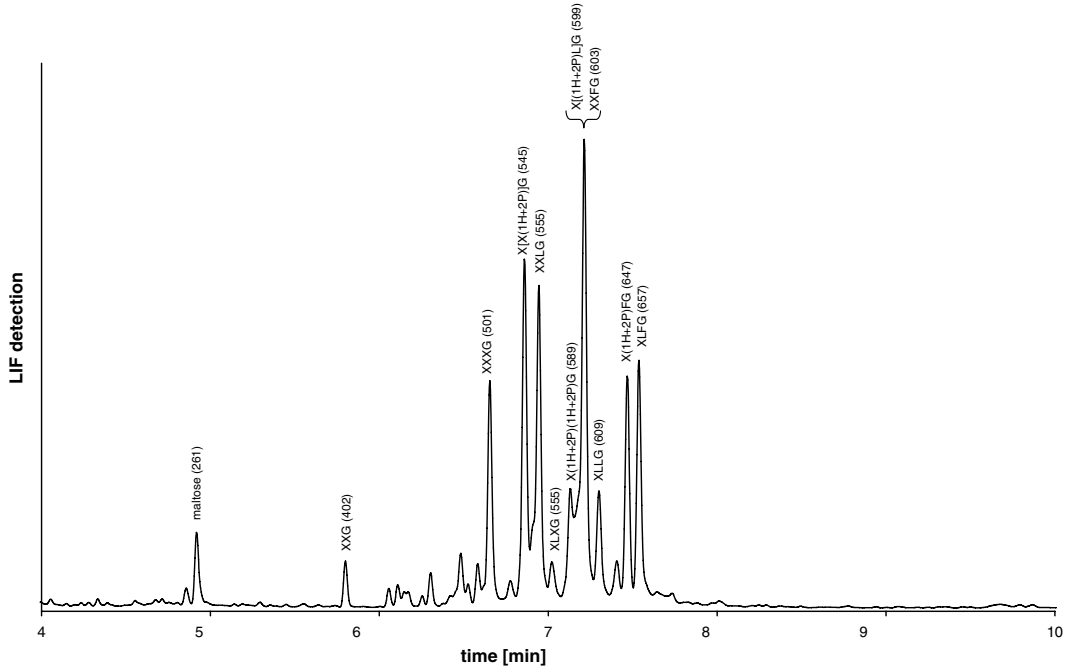

Figure 4a. CE-LIFD electropherograms of xyloglucan oligomers derived from bilberry CASS. Oligomers were identified by CE-MS according to their triple charged APTS oligomers (*m*/*z* between brackets). The side chain (1H+2P) was later identified as the U chain by NMR.

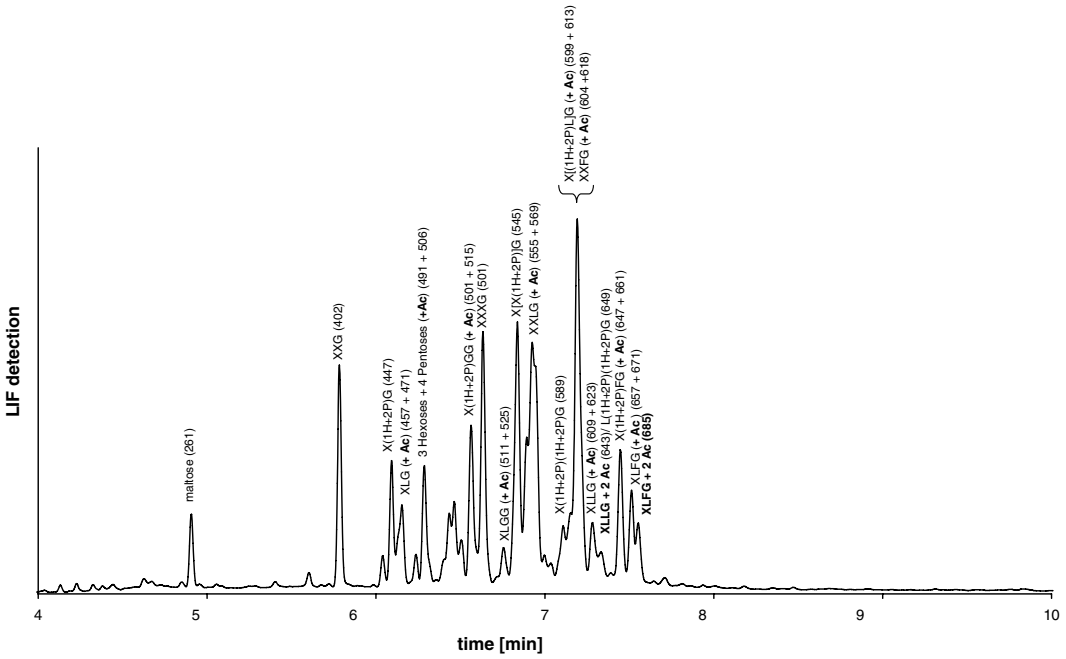

Figure 4b. CE-LIFD electropherograms of xyloglucan oligomers derived from bilberry AIS. Oligomers were identified by CE-MS according to their triple charged APTS oligomers (*m*/*z* between brackets). The side chain (1H+2P) was later identified as the U chain by NMR.

β-xylose containing side chain (U) or the arabinose containing side chain (S).[2] The position of the sugar residues in the oligomers was indicated by a letter (see structure in Fig. 3) that was used before for xyloglucan oligosaccharides,[23] starting with a c for the side chain on the nonreducing end.

The spectrum of XX(1H+2P)G showed the anomeric proton doublet of the β-D-xylose (δ 4.58, $J_{1,2}$ 7.37) forming the U-side chain. Furthermore, two unsubstituted α-D-xylose (δ 4.94, $J_{1,2}$ 3.53 and δ 4.96, $J_{1,2}$ 3.58) and one substituted α-D-xylose (δ 5.12, $J_{1,2}$ 3.66) were shown to be present. In the anomeric region, proton doublets of the other sugar residues were shown. The (1H+2P) side chain was identified as the U-side chain, which was previously reported in xyloglucans form the argan tree.[13] In X(1H+2P)(1H+2P)G, two anomeric proton doublets of β-D-xylose (δ 4.57, $J_{1,2}$ 7.35 and δ 4.57, $J_{1,2}$ 8.04) were seen. Each of them was probably linked to an α-D-xylose (δ 5.12, $J_{1,2}$ 3.60 and δ 5.15, $J_{1,2}$ 3.17), leaving the α-D-xylose[c] of the nonreducing end free (δ 4.95, $J_{1,2}$ 3.16). This oligomer contained two U side chains and was identified as XUUG. XL(1H+2P)G contained a β-D-xylose unit (δ 4.57, $J_{1,2}$ 7.89), as well. The β-D-galactose unit could be identified (δ 4.56, $J_{1,2}$ 7.56) and the β-D-galactose and the β-D-xylose were attributed to two substituted α-D-xyloses (δ 5.14, $J_{1,2}$ 3.15 and δ 5.163, $J_{1,2}$ 2.75). This oligomer was identified as XLUG. X(1H+2P)FG showed the typical α-L-fucose residue (δ 5.27, $J_{1,2}$ 3.70) and the substituted β-D-galactose (δ 4.61, $J_{1,2}$ 6.93) next to the β-D-xylose unit (δ 4.57, $J_{1,2}$ 7.40). The oligomer was identified to be XUFG.

2.8. Contribution of oligomeric building blocks to xyloglucan structure

To compare the composition of XEG digests from CASS and AIS, the peak area % of the different oligomers after separation by HPAEC (CarboPac PA 1 and CarboPac PA 100) and CE was calculated (Table 3). With HPAEC-PAD the area depends not only on the concentration, but on the response factors of different oligosaccharides,[24] as well. However, no big differences in response factors between the oligomers were expected, because the xyloglucan oligomers were of similar structure and size. In the HPAE-chromatogram of the CASS digest, XXUG was the main oligomer followed by XXFG, XXXG (probably coeluting with an oligomer tentatively assigned as XUGG), XXLG and XLFG. These oligomers were present to more than 10%. Comparing the elution patterns of the digest separated on two different columns, XXLG contributed with 11%, XLFG with 10%, and XLXG with less than 1% to the total area of xyloglucan oligosaccharides. While XXUG 2 and XLUG co-eluted on CarboPac PA 100, separation of CarboPac PA 1 showed the presence of these two oligomers in similar amounts. Similar to black currants,[17] the building block composition in CASS was the same as in CASS unbound and the not further described fractions from DEAE. When comparing the xyloglucan oligomers present in CASS with the oligomers present in AIS, different profiles of the xyloglucan oligomers were observed. From AIS more XXG-type oligosaccharides were released. Furthermore, more XXXG (probably coeluting with an oligomer tentatively assigned as XUGG) was

Table 2. ^{1}H NMR chemical shifts and coupling constants for reduced xyloglucan oligomers of bilberries, tentatively assigned according to the literature

Sugar residue	δ (ppm)	$J_{1,2}$
XXUGol		
β-Glc^c	4.54	8.56
β-Glc^b	4.58	7.43
β-Xyl^b	4.58	7.37
β-Glc^a	4.63	8.50
α-Xyl^c	4.94	3.53
α-Xyl^b	4.96	3.58
α-Xyl^a	5.12	3.66
XUUGol		
β-Glc^c	4.52	7.33
β-Glc^b	4.54	9.67
β-Xyl^a	4.57	7.35
β-Xyl^b	4.57	8.04
β-Glc^a	4.63	7.20
α-Xyl^c	4.95	3.16
α-Xyl^a	5.12	3.60
α-Xyl^b	5.15	3.19
XLUGol		
β-Glc^c	4.51	8.18
β-Glc^b	4.54	7.73
β-Gal^b	4.56	7.56
β-Xyl^a	4.57	7.89
β-Glc^a	4.63	7.19
α-Xyl^c	4.95	3.10
α-Xyl^{a,*}	5.14	3.15
α-Xyl^{b,*}	5.16	2.75
XUFGol		
β-Glc^b	4.49	7.74
β-Glc^c	4.54	7.35
β-Xyl^b	4.57	7.40
β-Gal^a	4.61	6.93
β-Glc^a	4.62	7.22
α-Xyl^c	4.95	3.13
α-Xyl^{b,*}	5.13	3.72
α-Xyl^{a,*}	5.15	2.91
α-Fuc^a	5.27	3.70

The positions of the different sugar residues are labelled by letters as described before.[13,23]

* May be interchanged.

present in the AIS digest. Less XXUG 1 and XUFG were determined instead. XXLG was the main oligosaccharide present in the XEG digest of bilberry AIS.

Using CE-LIFD rather similar results as by HPAEC-PAD were observed. Variations of the two methods were due to different response factors of HPAEC. In CE-LIFD, the area % composition is equal to the mol % composition due to labelling of the reducing end. Only the amount of XXUG in xyloglucan of CASS was present in a lower percentage when determined by CE-LIFD compared to HPAEC-PAD.

3. Discussion

Bilberry xyloglucans were analysed after degradation of xyloglucans with XEG. Compared to the known xyloglucan structures,[1,13,17] bilberry xyloglucans were more complex. HPAEC and CE were again shown to be powerful tools for the characterisation of xyloglucan oligosaccharides,[17] even when many oligosaccharides are present with similar structures. Both analytical techniques provided similar results according to the composition of xyloglucans and when used together give additional information about the structure.

The sugar composition of the isolated xyloglucans (CASS unbound) showed that xyloglucans contained galactose and fucose next to xylose and glucose. Arabinose was not present. However, MALDI-TOFMS, ESIMS^n, and HPAEC coupled off line to MALDI-TOFMS or ESIMS^n showed clearly the presence of xyloglucan oligomers carrying two pentoses on one glucose residue of the backbone. That these oligomers did not contain an arabinose containing side chain (S) was confirmed by HPAEC, when xyloglucan oligomers derived from olives[2] were analysed under the same conditions. The retention times of the S-side chain containing oligosaccharides were much higher than the retention times of the xyloglucan oligomers in the bilberries. Further indications for the structure of the unknown side chain were found in the literature, where a regular and similar structure of xyloglucans is described for taxonomically related species.[1,2] Bilberries belong to the family of Ericaceae, order Ericales. The xyloglucan structure of one other member of Ericales has been described before: the argan tree (*A. spinosa* (L.) Skeels), which belongs to the family of Sapotaceae.[13] In these xyloglucan, the XUFG building block was identified and XUXG and XULG were proposed for the corresponding masses.

By ESIMS^2 analysis, we were able to locate the position of the side chain consisting of two pentoses connected to one hexose. If the pentoses were connected to each other or both to the hexose had to be determined by ^{1}H NMR analysis of four isolated oligomers. All four contained a β-D-xylose attached to an α-D-xylose (the U side chain). In bilberry xyloglucans, XUG, XXUG, XUXG, XULG, XLUG, XUFG and XUUG were identified. XUG, XUUG, XXUG and XLUG are novel xyloglucan building blocks not described before. All four oligomers contained a β-D-xylose residue, which was unsubstituted and attached to an α-xylose residue.

Furthermore, acetylation of xyloglucan oligosaccharides obtained from AIS was shown. The acetyl groups are present on the galactose units of xyloglucan, as already shown in xyloglucan from sycamore cells.[25] Major differences in the composition of xyloglucans present in CASS and degradable in AIS were seen. AIS contained more XXG-type oligosaccharides, which are known to be present in these xyloglucan domains.[6] These differences are due to the different accessibility of xyloglucan domains.[17] In AIS mainly xyloglucans that cross-link celluloses or that are present in free loops are degraded, while in CASS xyloglucans that are hydrogen bound to

Table 3. Quantification of oligomers derived from xyloglucans of bilberries as analysed on HPAEC PA100, HPAEC PA1 and CE

	$M+Na^+$	$M+APTS^{3-}$	HPAEC PA100		HPAEC PA1		CE	
	m/z	*m/z*	CASS	AIS	CASS	AIS	CASS	AIS
XXG	791	403	2	8	2	7	1	6
XUG	923	447	1	2	—	—	1	4
XLG	953	457	2	6	1	6	—	4
3 Hexose, 4 pentose	1055	491	—	—	—	—	1	4
XXXG	1085	501	11[a]	15[a]	11[a]	15[a]	9	9
XUGG	1085	501	—	—	—	—	2	6
XLGG	1115	511	—	—	—	—	1	2
XXUG 1	1217	545	17	9	18	11	13[b]	10[b]
XXUG 2	1217	545	8[c]	9[c]	3	3	—	—
XLXG	1247	555	—	—	0	1	2	1
XXLG	1247	555	11	17	—	—	13	18
[XG]FG	1261	559	1	2	1	1		
XUUG	1349	589	5	2	5	2	5	3
XLUG	1379	599	—	—	3	8	—	—
X[LU]G	1379	599	2	0	—	—	—	—
XXFG	1393	603	12	11	14	12	19[d]	16[d]
XLLG	1409	609	4	2	5	3	6	3
LUUG	1511	643	1	0	1	1	2	2
XUFG	1525	647	9	4	11[e]	5[e]	9	5
LLUG	1541	653	5[f]	5[f]	4[f]	6[f]	—	—
XLFG	1555	657	11[g]	8[g]	19[h]	20[h]	10	5

The oligomers were identified by their masses, off line by MALDI-TOFMS and ESIMS for HPAEC and online with ESIMS for CE (area %).
[a] Coelution with XUGG.
[b] Coelution of all X[XU]G oligomers.
[c] Coelution with XLUG.
[d] Coleution with all X[UL]G.
[e] Coelution with X[LU]G.
[f] Coelution with XULG and XXUG 3.
[g] Coelution with XLXG.
[h] Coelution with XXLG.

cellulose are extracted, as well.[6] Perhaps the acetylation of the galactose side chain may also have an effect on the degradation of xyloglucan in AIS,[17] which would result in the observed differences in oligosaccharide composition.

In bilberry xyloglucans, small amounts of reduced oligomers (XXUGol and XXLGol) were shown to be present. They may be artefacts of the isolation procedure. However, they were present in xyloglucan from AIS, where the isolation procedure was mild and should not lead to the chemical modification of oligomers. If these oligosaccharide–alditols were present in native xyloglucan, they can only be present on the reducing end.

4. Conclusions

Bilberries contain xyloglucans of XXXG-type structure, although different XXG-type oligomers are released in small amounts from AIS. XXG-type oligomers are known to be present in xyloglucan domains that are less tightly bonded to cellulose.[6,26] Next to the galactose (L) and the fucose–galactose (F) containing side chain, the β-D-xylose containing side chain (U) was identified. The galactose residues were partly acetylated. Bilberry xyloglucans were shown to have one of the most complex structures described in the literature until now. They consist of more than 20 different building blocks, among them four novel ones. The presence of the U-side chain in seven xyloglucan oligomers from bilberries was proven, showing that the U-side chain is a common element of xyloglucans in the order Ericales.

5. Experimental

5.1. Material

Bilberries (*V. myrtillus* L.) were obtained from Kiantama Ltd, Suomussalmi, Finland. Alcohol insoluble solids (AIS) were prepared from homogenised berries. In three extraction steps (hot buffer, chelating agent, 50 mM sodium hydroxide), the pectic polysaccharides were extracted from AIS. The hemicelluloses were extracted from the residual cellulose with 6 M sodium hydroxide (concentrated alkali soluble solids; CASS) as described previously.[15]

5.2. Fractionation of hemicelluloses with anion exchange chromatography

About 250 mg CASS was dissolved in water and the solution was centrifuged (10,000*g*). The supernatant

was applied on a DEAE Sepharose Fast Flow column (100 × 2.6 cm, GE Healthcare, Chalfont St. Giles, United Kingdom) and the unbound material was eluted with 530 mL of 5 mM sodium acetate at pH 5.0 (CASS unbound).[18]

5.3. Degradation of xyloglucan with xyloglucan specific *endo*-glucanase (XEG)

Xyloglucan containing material (5 mg) was dissolved in 1 mL of 50 mM sodium acetate buffer (pH 5.0) and incubated with 1 μL xyloglucan specific *endo*-glucanase (XEG, EC 3.2.1.151 from *Aspergillus aculeatus*, 2259 U/mL)[16] overnight. AIS (5 mg/mL) were incubated with additional 5 μL polygalacturonase (EC 3.2.1.15 from *Kluyveromyces fragilis*, 16 U/mL)[27] and 1 μL pectin methyl esterase (EC 3.1.1.11 from *Aspergillus niger*, 180 U/mL)[28] for pectin degradation.[2]

5.4. Sugar composition as alditol acetates

For determining the sugar composition, samples were pre-hydrolysed using 72% w/w sulfuric acid at 30 °C for 1 h and subsequently hydrolyzed with 1 M H_2SO_4 at 100 °C for 3 h.[29] Afterwards the sugars were derivatised to their alditol acetates and determined by gas chromatography[30] using inositol as internal standard.

5.5. Uronic acid content

The total uronic acid content was determined photometrically with the automated *m*-hydroxydiphenyl assay.[31]

5.6. HPAEC of xyloglucan oligomers

Xyloglucan oligosaccharides were analysed on a CarboPac PA 100 column (4 × 250 mm, Dionex, Sunnyvale, USA) and on a CarboPac PA 1 column (2 × 250 mm, Dionex, Sunnyvale, USA) with pulse amperometric detection (PAD) using a column specific sodium hydroxide–sodium acetate-gradient as described previously[32] and a flow of 1 mL/min for the 4 × 250 mm column and of 0.3 mL/min for the 2 × 250 mm column, respectively.

For desalting a FC-203B fraction collector (Gilson, Middleton, USA) was connected that collected fractions of 30 s directly after the PAD. These fractions were collected in a Sep-Pak tC18 (40 mg) 96-well plate (Waters, Milford, USA) containing 0.5 mL of 0.5 M acetic acid per well. The eluent was sucked through the Sep-Pak tC18 (40 mg) 96-well plate by vacuum with a MultiScreen resist vacuum manifold (Millipore, Billerica, USA). After washing two times with 1 mL water, the oligomers were eluted with 2 mL methanol and the fractions were dried at 60 °C.[17]

Preparative HPAEC was performed on a preparative CarboPac PA 100 column (22 × 250 mm, Dionex, Sunnyvale, USA). The same gradient as on analytical scale was applied with a flow of 18 mL/min. Fractions were collected every 30 s.

5.7. MALDI-TOFMS

Matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOFMS) was performed using an Ultraflex workstation (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser of 337 nm and operated in a positive mode. After a delayed extraction time of 200 ns, the ions were accelerated to a kinetic energy of 12,000 V and detected using reflector mode. The lowest laser power required to obtain good spectra was used and at least 100 spectra were collected. The mass spectrometer was calibrated with a mixture of maltodextrins (mass range 350–2350).

The xyloglucan oligomers were desalted using Sep-Pak tC18 (20 mg) cartridges (Waters, Milford, USA).[17] The sample solution (2 μL) was mixed on the MALDI-TOF-plate (Bruker Daltonics, Bremen, Germany) with 2 μL matrix solution of 9 mg/mL 2,5-dihydroxybenzoic acid (Bruker Daltonics, Bremen, Germany) in 30% acetonitrile and dried under a stream of air.[33]

5.8. ESIMS^n

Electrospray ionisation mass spectrometry (ESIMS) was performed on a LTQ Ion-trap (Thermo Electron, San Jose, USA). The sample was applied through a PicoTip emitter capillary (4 μm ID of the tip, New Objective, Woburn, USA). MS analysis was carried out in the positive mode using a spray voltage of 1.5 kV and a capillary temperature of 200 °C. The instrument was autotuned on xyloglucan oligosaccharides. MS^2 and higher were performed using a window of 1 *m*/*z* and a relative collision energy of 30%.[17]

5.9. CE-LIFD

For capillary electrophoresis (CE), xyloglucan oligosaccharides were labelled with 8-aminopyrene-1,3,6-trisulfonate (APTS) using the ProteomeLab Protein Characterisation kit (Beckman Coulter, Fullerton, USA).

The labelled oligosaccharides were separated on a polyvinyl alcohol (N CHO) coated capillary (50 μm ID × 50.2 cm, detector after 40 cm, Beckman Coulter, Fullerton, USA) using a ProteomeLab PA 800 characterisation system (Beckman Coulter, Fullerton, USA) and a laser induced fluorescence detector (LIFD) at an excitation wavelength of 488 nm and an emission wavelength of 520 nm (Beckman Coulter, Fullerton, USA). The separation was carried out in reversed polarity at

30 kV in a 25 mM acetate buffer containing 0.4% polyethylene oxide at pH 4.75. The capillary was kept at 25 °C.[17]

5.10. CE-ESIMS^n

For identification of the different oligomers, separation was carried out on a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, USA) with a fused silica capillary (50 μm ID × 80 cm) connected to a UV-detector (after 20 cm) and an ESIMS detector (LTQ Ion-trap, Thermo Electron, San Jose, USA) by a device made in our laboratory. The separation was carried out in reversed polarity at 28 kV in a 50 mM ammonium acetate buffer containing 0.2% formic acid at pH 2.8. The capillary was kept at 15 °C. For ESIMS, a sheet flow of 4 μL/min of 75% isopropanol in water was used. ESIMS was operated in the negative mode using a spray voltage of 2.2 kV and a capillary temperature of 190 °C. The instrument was auto-tuned on xyloglucan oligosaccharides. MS^2 and higher was performed using a window of 1 m/z and a relative collision energy of 30%.[17]

5.11. ^{1}H NMR spectroscopy

Prior to NMR analyses, the samples were reduced with sodium borohydrate,[23] desalted using Sep-Pak tC18 (20 mg) cartridges (Waters, Milford, USA) and the reduced xyloglucan oligomers were dissolved in 99.96% D_2O (Cambridge Isotope Laboratories, USA) and after freeze-drying dissolved in 99.996% D_2O (Cambridge Isotope Laboratories, USA). ^{1}H NMR spectra were recorded at a probe temperature of 300 K on a AMX-500 spectrometer (Bruker BioSpin, Rheinstetten, Germany) located at the Wageningen NMR Centre. Chemical shifts were expressed in ppm relative to internal acetone: 2.225 ppm. The 1D ^{1}H proton spectra were recorded with pre saturation of the HOD signal (70 dB) at 500.13 MHz using 256 scans of 8192 data points and a sweep width of 6000 Hz.

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