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A comparison of liquid chromatography, capillary electrophoresis, and mass spectrometry methods to determine xyloglucan structures in black currants

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Abstract

Different separation (HPAEC, RP–HPLC, CE) and identification (MALDI–TOF–MS, ESI–MS^n) techniques were compared to analyse oligosaccharides obtained after incubation of xyloglucan with endo-glucanase. It was possible to analyse xyloglucan oligosaccharides with each technique. Several techniques, including off line (HPAEC–MALDI–TOF–MS) or online (CE–ESI–MS^n, RP–HPLC–ESI–MS^n) connection provided complementary information on xyloglucan structure. Online CE–MS and RP–HPLC–MS are described for the first time in xyloglucan analysis. Advantages and disadvantages of the techniques for different purposes such as structural characterisation of oligosaccharides or oligosaccharide profiling are discussed. Black currant xyloglucans had a rather simple XXXG-type structure with galactose and fucose containing side chains.

Keywords: Ribes nigrum; Black currant; Cell wall polysaccharides; Xyloglucan; CE–MS; HPLC–MS; HPAEC; MALDI–TOF–MS

1. Introduction

Xyloglucans belong to the group of hemicelluloses and were first described in tamarind seeds, where they function as storage polysaccharides [1]. Later xyloglucans were also described in plant cell walls [2] and since then their structure and the role in the plant cell wall have been studied intensively. Today it is known that xyloglucan is the main hemicellulose of dicotyledons and its composition is dependent on the taxonomic family [3,4].

Xyloglucans cover and interlink cellulose microfibrils [5,6]. In the plant cell wall's cellulose–xyloglucan network three xyloglucan domains were described: one domain is present in free loops or cross links and can be degraded by endo-glucanases, a second domain covers the cellulose microfibrils and can be extracted with concentrated alkali, and a third domain is entrapped within the amorphous cellulose microfibrils and can only be accessed after degradation of cellulose [6–8].

The backbone of xyloglucan is a β-1,4-linked glucan chain [9] to which different short side chains are attached [2]. To simplify the nomenclature, the different side chains of xyloglucan were assigned one-letter codes such as G for unsubstituted glucose and X for a xylose attached to the glucose [10]. Xyloglucan can be classified in XXXG-type (Fig. 1), XXGG-type (typical for *Solanaceae*), and XXXGG-type structures [11,12]. XXXX-type xyloglucan of which every glucose is substituted with arabinose in position 2 was also reported [13]. Molecular modelling showed that the fucose-galactose containing side chain (F) is important for flattening of the back bone in solution and, therefore, initiates xyloglucan-cellulose binding [14]. However, fucose is not required for cellulose binding [15].

Black currants are an important crop of northern Europe and are mainly used for juice production. In our recent studies, cell wall polysaccharides of black currants were characterised in detail [16,17]. The hemicellulose rich fraction of black currants contained a high amount of xylose and glucose, indicating the presence of xyloglucan. The structure of xyloglucan is regular and similar within one botanic family [3]. Taxonomic information of black currants was obtained from the NCBI database [18]. Black currants belong to the family of *Grossulariaceae*, order *Saxifragales*. The structure of xyloglucan of neither a member of the same family nor of the same order has been reported in literature until now.

High performance anion exchange chromatography (HPAEC) is the most common technique for separation of

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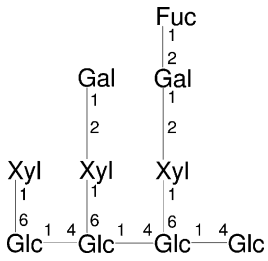

Fig. 1. Xyloglucan oligomer XLFG as example for XXXG-type xyloglucan.

xyloglucan oligomers [19]. Matrix assisted laser desorption time of flight mass spectrometry (MALDI–TOF–MS) together with post source decay (PSD) was used for determination of the mass and structure of different oligomers [3,4,20,21]. While oligosaccharides were derivatised to their acetylated form and analysed by fast-atom bombardment mass spectrometry (FAB-MS) in the past [22], the development of improved ionisation techniques and more sensitive electrospray ionisation mass spectrometers (ESI–MS^n) made it possible to use MS and MS^n of underivatised oligomers to unravel their structure [23]. Instead of HPAEC, reversed phase high performance liquid chromatography (RP–HPLC) has also been used for determination of xyloglucan oligomers [24] allowing online ESI–MS^n-detection. Recently capillary electrophoresis (CE) of labelled xyloglucan oligomers was performed and the peaks were identified by comparing them to isolated fractions from HPAEC [20]. To prove the presence of xyloglucans and to characterise them, xyloglucans of black currants were degraded by xyloglucan specific endo-glucanase (XEG) and the obtained oligomers were analysed using a number of different techniques.

The aim of this study was to give an overview of state of the art techniques that are suitable for xyloglucan analysis. Therefore, xyloglucan is degraded by endo-glucanase and the obtained oligomers are analysed for oligosaccharide profiling and structural characterisation of the oligosaccharides. The advantages and disadvantages of the different techniques for these two purposes are exemplified on black currant xyloglucans, which have not been characterised before.

2. Materials and methods

2.1. Material

Black currants (*Ribes nigrum* L.) were obtained from Kiantama Ltd., Suomussalmi, Finland. Alcohol insoluble solids (AIS) were prepared from homogenised berries. In three extraction steps 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). The solution was directly neutralised in an ice bath, subsequently dialysed and freeze dried [17].

2.2. Fractionation of hemicelluloses with anion exchange chromatography

About 250 mg CASS were dissolved in water and centrifuged. The pellet was collected (CASS residue) and the supernatant was fractionated on a DEAE Sepharose Fast Flow column (100 cm × 2.6 cm, GE Healthcare, Upsala, Sweden) into three fractions: unbound material was eluted with 530 mL 5 mM sodium acetate at pH 5.0 (CASS unbound), the second fraction was eluted with 530 mL 2 M sodium acetate buffer at pH 5.0 (CASS NaOAc), and the last fraction was eluted with 530 mL 0.5 M sodium hydroxide (CASS NaOH) [25].

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

Xyloglucan containing material (5 mg) was dissolved in 1 mL 50 mM sodium acetate buffer (pH 5.0) and incubated with 1 μL xyloglucan specific endo-glucanase (XEG, EC 3.2.1.4 from *Aspergillus aculeatus*, 2259 U/mL) over night. AIS (5 mg/mL) were incubated with additional 5 μL polygalacturonase (EC 3.2.1.15 from *Kluyveromyces fragilis*, 16 U/ml) and 1 μL pectin methyl esterase (EC 3.1.1.11 from *Aspergillus niger*, 180 U/ml) for pectin degradation [4].

2.4. Sugar composition as alditol acetates

For determining the sugar composition, samples were pre-hydrolysed using 72%, w/w sulphuric acid at 30 °C for 1 h and subsequently hydrolyzed with 1 M sulphuric acid at 100 °C for 3 h [26]. Afterwards the sugars were derivatised to their alditol acetates and determined by gas chromatography [27] using inositol as internal standard.

2.5. Uronic acid content

The total uronic acid content was determined photometrically with the automated *m*-hydroxydiphenyl assay [28].

2.6. HPAEC of xyloglucan oligomers

Xyloglucan oligosaccharides were analysed on a CarboPac PA 100 column (4 mm × 250 mm, Dionex, Sunnyvale, USA) and on a CarboPac PA 1 column (2 mm × 250 mm, Dionex, Sunnyvale, USA) with pulse amperometric detection (PAD) using a column specific sodium hydroxide-sodium acetate-gradient as described before [19] and a flow of 1 mL/min for the 4 mm × 250 mm column and of 0.3 mL/min for the 2 mm × 250 mm column, respectively.

Desalted fractions were obtained in two ways:

(a) The ultra-self-regenerating anion suppressor 4 mm-unit (ASRS, Dionex, Sunnyvale, USA) was connected after the PAD to exchange the sodium ions for hydronium ions (H_3O^+) followed by the ultra-self-regenerating cation suppressor 4 mm-unit (CSRS, Dionex, Sunnyvale, USA) to

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exchange the acetate ions for hydroxide ions (OH^-). The continuous desalting of the eluent was achieved by the electrolysis of demineralised water (8 ml/min) in both suppressors. Fractions of 30 s were collected in a 96-well plate using a FC-203B fraction collector (Gilson, Middleton, USA) and freeze dried [29].

(b) Directly after the PAD the FC-203B fraction collector (Gilson, Middleton, USA) was connected collecting fractions of 30 s. These fractions were collected in a Sep-Pak tC18 (40 mg) 96-well plate (Waters, Milford, USA) containing 0.5 mL 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 2 times with 1 mL water, the oligomers were eluted with 2 mL methanol and dried at 60 °C.

2.7. MALDI–TOF–MS

Matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI–TOF–MS) was performed using an Ultraflex workstation (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser of 337 nm and operated in positive mode. After a delayed extraction time of 200 ns, the ions were accelerated to a kinetic energy of 12,000 V. The ions were 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 300–2500).

Two microliters sample solution were mixed on the MALDI-TOF-plate (Bruker Daltonics, Bremen, Germany) with 2 μL matrix solution of 9 mg/mL 2,5-dihydroxy-benzoic acid (Bruker Daltonics, Bremen, Germany) in 30% acetonitrile and dried under a stream of air [30].

2.8. Post-source decay

For post source decay (PSD) the same conditions as for MALDI–TOF–MS were used. PSD was recorded in the positive mode. The spectrum was dived into different independently measured segments, which were put together to one PSD spectrum by the software FlexAnalysis (Bruker Daltonics, Bremen, Germany) [30].

2.9. ESI–MS^n

Electrospray ionisation mass spectrometry (ESI–MS) 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, 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%.

2.10. RP–HPLC–ELSD–ESI–MS^n

Xyloglucan oligomers were separated by reversed phase HPLC (RP–HPLC) on a XTerra MS C18 3.5 μm column (4.6 mm × 150 mm, Waters, Milford, USA) with a linear gradient from 0 to 15% of methanol in 100 min followed by a 10 min washing step with 100% methanol at a flow of 0.9 mL/min. After post column splitting 3/4 of the eluent went to a evaporate light scattering detector ELSD 2000 (Alltech, Lexington, USA) and 1/4 went to a LCQ Deca XP MAX ESI–MS^n detector (Thermo Electron, San Jose, USA). ESI–MS^n was operated in positive mode using a spray voltage of 4.0 kV and a capillary temperature of 200 °C and 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%.

2.11. 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 of 488 nm and an emission of 520 nm (Beckman Coulter, Fullerton, USA). The separation was carried out in reversed polarity at 30 kV in a 25 mM sodium acetate buffer containing 0.4% polyethylene oxide at pH 4.75. The capillary was kept at 25 °C. APTS labelled maltose was used as internal standard.

2.12. CE–ESI–MS^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 and an ESI–MS^n detector (LTQ Ion-trap, Thermo Electron, San Jose, USA) by a device made in our laboratory (Fig. 2). The separation was carried out at 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 ESI–MS^n a sheath flow of 4 μL/min of 75% isopropanol in water was used. ESI–MS^n was operated in the negative mode using a spray voltage of 2.2 kV and a capillary temperature of 190 °C and 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%.

3. Results and discussion

Xyloglucans from black currants were analysed following two different approaches. In the first approach, hemicelluloses were extracted with 6 M sodium hydroxide from cellulose microfibrils after removal of pectins (concentrated alkali soluble

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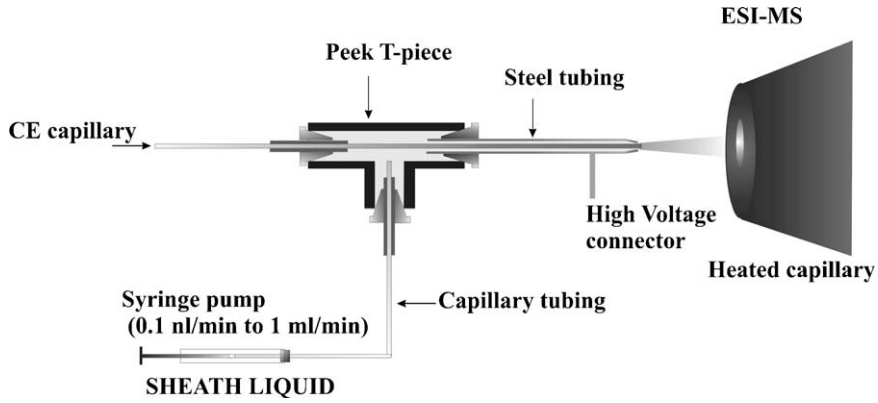

Fig. 2. Schematic view of CE–MS device.

solids; CASS). Xyloglucans were further purified on an anion exchange column. Neutral hemicelluloses including xyloglucan were separated from anionic hemicelluloses and pectins. However, possible acetyl esters were saponified during alkali extraction and could not be determined by this approach. Thus, to analyse a possible acetylation pattern of xyloglucan, xyloglucans were degraded with xyloglucan-specific endo-glucanase (XEG) directly in alcohol insoluble solids (AIS) in a second approach [4].

3.1. Yield and sugar composition of xyloglucan containing fractions

Sugar compositions of cell wall fractions indicate the polysaccharides present. In CASS xylose, glucose, and mannose were the major sugar residues (Table 1). The high amount of xylose present in a 1.6:1 ratio to glucose indicated the presence of xylans next to xyloglucans. Uronic acid was present in small amounts. These uronic acids can be glucuronic acids from glucuronoarabinoxylans or galacturonic acids from pectins [17]. CASS were hardly soluble in water: 83% remained insoluble (CASS residue). The dissolved polysaccharides were loaded on an anion exchange column. The smallest part of them did not bind to the column and was eluted with water (CASS unbound). More polysaccharides were eluted with 2 M sodium acetate (CASS NaOAc) and 0.5 M sodium hydroxide (CASS NaOH). CASS unbound contained xylose and glucose in a ration 2:4. The amounts of mannose and galactose were also high showing that next to xyloglucans galactomannans were present [17]. The high amount of fucose suggested a high percentage of fucosilated xyloglucan (*vide infra*). In CASS NaOAc and CASS NaOH, xylose was by far the major sugar moiety. High amounts of uronic acids and high amounts of arabinose and mannose were present, as well. CASS NaOH and CASS NaOAc differed in the content of glucose: while CASS NaOAc contained only 5 mol%, CASS NaOH contain 10 mol%. These two fractions were rich in charged xylans and contained some galactomannans and maybe pectins. CASS residue contained xylose and glucose in a ratio of 2.4:4 accompanied by fucose. Additionally, mannose and galactose residues were present. Mainly xyloglucan and galactomannan and maybe some xylans were present in the CASS residue. AIS of black currants contained mannose, glucose, and uronic acid as major sugar residues. Xylose was present in minor quantities.

The main hemicelluloses present in black currants were xyloglucans, xylans, and galactomannans. However, sugar composition can only indicate the presence of these polysaccharides, but for proving the presence of the different hemicelluloses other analytical approaches including enzyme degradation are necessary.

3.2. HPAEC

Endo-glucanase II is a xyloglucan specific endo-glucanase (XEG), which is known to split xyloglucan between an unsubstituted glucose and a xylose-substituted glucose residue [31]. By this digestion xyloglucan can be degraded into specific oligosaccharide building blocks of xyloglucan [10].

The chromatograms of black currant xyloglucan oligomers separated on two different HPAEC columns are shown in Fig. 3. The different oligomers were identified by comparing the retention time with other known xyloglucan oligosaccharides from

Table 1
Sugar composition of xyloglucan containing fractions of black currants (mol %)

	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	Total sugars (w/w %)	Yield
CASS total	1	2	6	37	16	8	23	8	63	4% of AIS
CASS unbound	0	9	3	16	17	11	38	7	46	4% of CASS
CASS NaOAc	2	0	14	42	13	8	5	17	24	7% of CASS
CASS NaOH	2	1	10	42	10	6	10	20	8	6% of CASS
CASS residue	0	4	2	22	20	12	37	3	64	83% of CASS
AIS	2	0	11	6	13	6	20	41	38	9% of fresh berries

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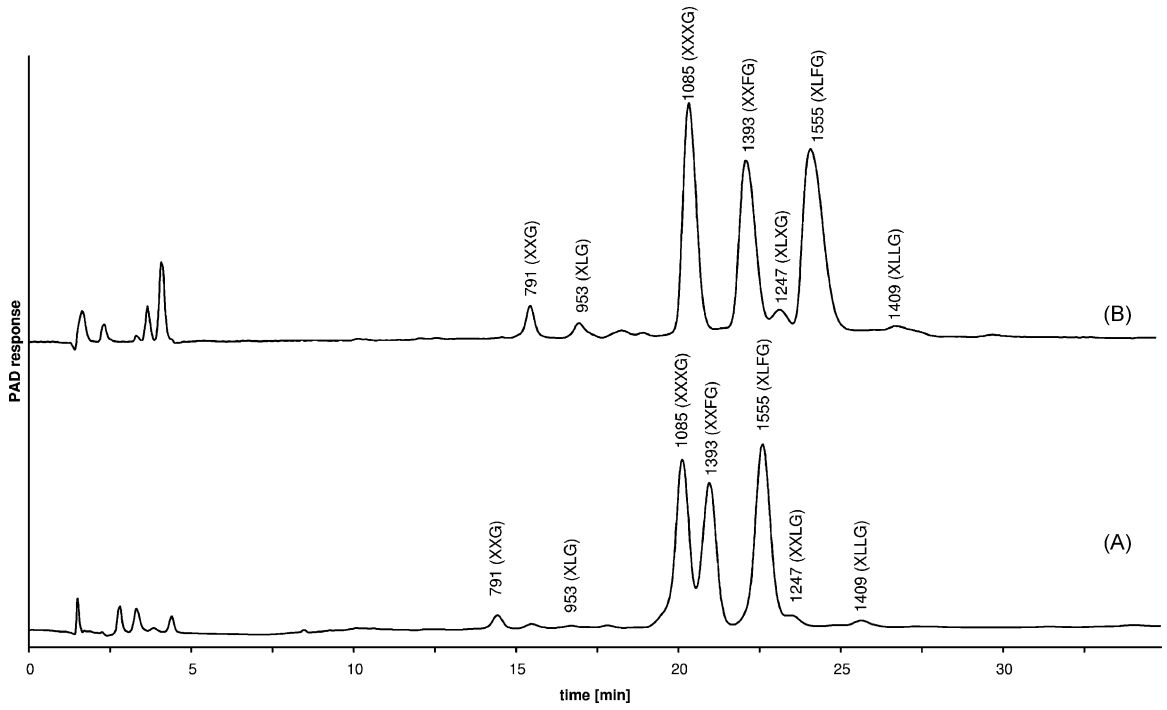

Fig. 3. Xyloglucan oligomers obtained after degradation with XEG on HPAEC with a 2 mm Dionex PA1 (A) and a 4 mm Dionex PA100 (B) column.

tamarind [19] olive [4] and soy [25] and, additionally, by off-line MALDI–TOF–MS and ESI–MS^n. For MALDI–TOF–MS and ESI–MS^n sample solutions were desalted. In one desalting procedure tested, two desalting membranes were connected after the detector to remove anions and cations from the eluting fractions [29]. With this method it was possible to identify the peaks in the elution pattern using MALDI–TOF–MS and ESI–MS^n, but peak broadening was observed and the retention time from the PAD was delayed because of tubing dead volume of the membrane devices. These problems were solved in the second desalting procedure by using a C18-solid phase extraction (SPE) 96-well plate for fraction collection. The fractions were collected directly after the PAD. After SPE, the final fractions were salt free and, additionally, peak broadening was prevented. By identifying the peaks it was shown that the CarboPak PA 1 column separated XXLG from XLFG, but not XLXG and XLFG, the CarboPak PA 100 column separated XLXG and XLFG, but not XXLG and XLFG.

Black currant xyloglucans consisted of three main and four minor oligomers. Xyloglucan oligomers of black currants carry galactose (L) and fucose-galactose (F) containing side chains. Only by using the two different HPAEC columns and off line ESI–MS^n, it was possible to show that both XLXG and XXLG were present. Xyloglucan oligomers were shown in XEG digests of both the unbound (neutral) and bound (charged) fractions obtained from anion exchange chromatography of CASS, although neutral polysaccharides such as xyloglucans should not bind to an anion exchanger. This behaviour was shown before and was explained by a proposed covalent cross link of the reducing glucose of xyloglucan to pectic galactan [5].

Table 2
Oligomers present in xyloglucan of black currants as calculated from HPAEC with a Dionex PA 1 (A) and a Dionex PA 100 (B) column

	XXG (Area %)	XXXG (Area %)	XXFG (Area %)	XLXG (Area %)	XLFG (Area %)	XXLG (Area %)	XLLG (Area %)
(A) PA1							
CASS total	1	32	26	–	37	2	1
CASS unbound	1	31	27	–	40	1	1
CASS NaOAc	1	30	24	–	40	2	2
CASS NaOH	1	31	26	–	39	2	2
CASS residue	2	32	26	–	36	3	2
AIS	3	34	28	–	30	5	1
(B) PA 100							
CASS total	3	31	27	4	34	–	1
CASS unbound	2	32	26	2	37	–	1
CASS NaOAc	3	29	24	2	40	–	2
CASS NaOH	2	31	25	2	38	–	1
CASS residue	4	23	28	6	36	–	4
AIS	5	33	27	5	27	–	2

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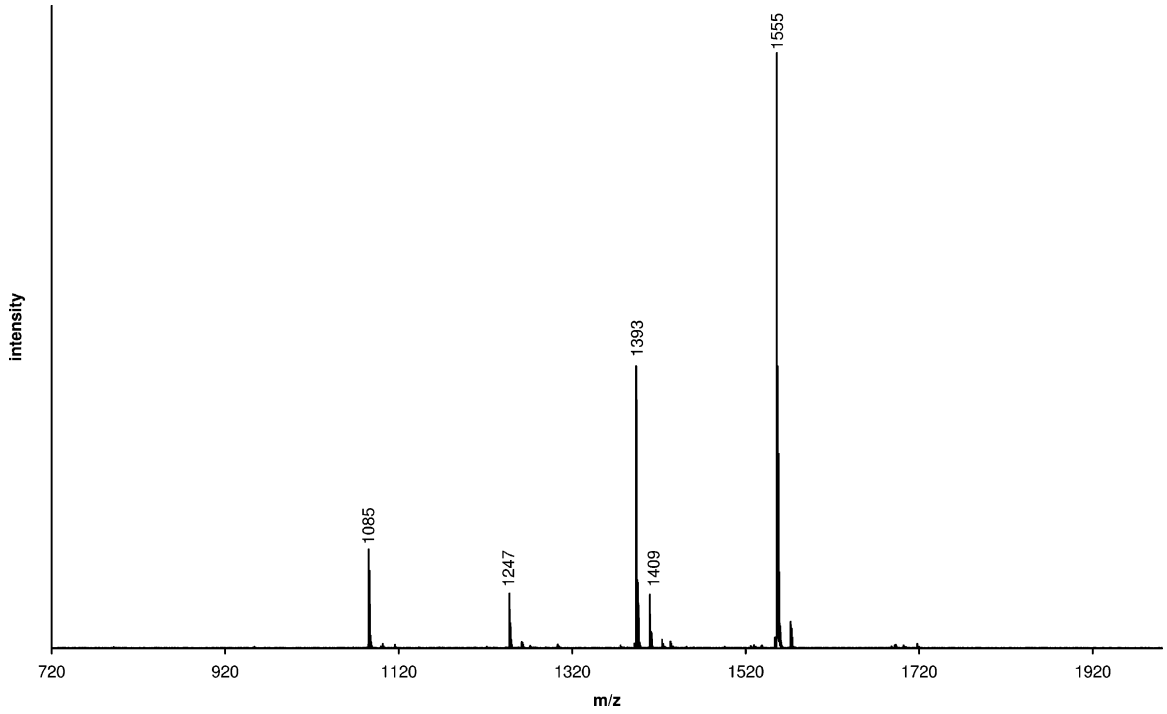

Fig. 4. MALDI–TOF mass spectrum of xyloglucan oligomers obtained after XEG treatment of black currant CASS (only sodium adducts are labelled).

Relative amounts of the different xyloglucan oligomers in the hemicellulose containing fractions are shown in Table 2. CASS, CASS unbound, CASS NaOAc, CASS NaOH, and CASS residue showed no significant differences. XLFG was the major oligomer followed by XXXG and XXFG. In the xyloglucan oligomers derived from AIS more minor oligomers were present compared to digests of CASS and fractions derived from them. Although the solubility and elution behaviour on anion exchange chromatography of xyloglucans extracted with 6 M alkali (CASS and fractions derived from them) was different, the relative amounts of oligomers present were the same. Probably not the structure of xyloglucan determines the elution behaviour, but the binding of xyloglucan to other (charged) polysaccharides like xylans or pectins. This is in agreement with a proposed covalent cross link between xyloglucans and pectins [5,32,33].

The relative proportions of the different xyloglucan oligomers differed in black currant AIS compared to CASS and the fractions derived from it. After degradation of xyloglucan in AIS, XXXG was the main oligomer, followed by XLFG and XXFG in the same amounts. XLXG, XXLG, and XLLG were minor oligomers. The accessibility of xyloglucan can explain these findings. In AIS only the xyloglucan that cross links celluloses or forms free loops is available for XEG [7]. Another xyloglucan domain is still attached to cellulose and cannot be degraded in AIS, but will be extracted with concentrated alkali [7]. A possible acetylation of the galactose unit as shown in sycamore xyloglucan [34] might be another explanation for a difference in degradability of xyloglucan domains. Acetylation of the galactose residues xyloglucans in AIS might partly hinder enzymatic degradation of cellulose bound xyloglucan. However, only acetylation of glucose residues of the backbone inhibits degradation by XEG [35].

HPAEC is a robust method to analyse oligosaccharide profiles of xyloglucan. The possibility to couple HPAEC off line to mass spectrometers allows identification of unknown oligomers. Especially the use of SPE for desalting simplifies the off line coupling. A disadvantage is the necessity for two different columns to separate all oligomers and difficult absolute quantification due to different response factors by PAD of mono and oligomers [36].

3.3. MALDI–TOF–MS

Oligosaccharides obtained by degradation of xyloglucan with XEG can be quickly identified by the mass to charge ratios of their sodium adducts with MALDI–TOF–MS. A comparison of saponified oligomers, as obtained from the alkali extract, with non-saponified oligomers, as obtained from AIS, shows acetylation of oligomers by a m/z difference of 42 to the oligomer without an acetyl group. Prior to analysis, solid phase extraction (SPE) can be used to desalt the enzyme digest of xyloglucans and to remove pectic oligomers from it [4]. This is an elegant way to remove interfering charged oligosaccharides.

The mass spectrum of xyloglucan oligosaccharides derived from CASS showed three major and two minor peaks (Fig. 4). The three major peaks belonged to the single charged sodium adducts of XXXG ($m/z = 1085$), XXFG ($m/z = 1393$), and XLFG ($m/z = 1555$). These oligomers were present in the highest amounts as determined by HPAEC. Small peaks of potassium adducts were present for all oligomers, even the minor ones. The m/z of 1409 is the m/z of either the sodium adduct of XLLG or the potassium adduct of XXFG. Probably both were present, but by comparing the ratio of sodium to potassium adducts of the other oligomers it can be concluded that the major part of the peak was the sodium adduct of XLLG. The other small peak was

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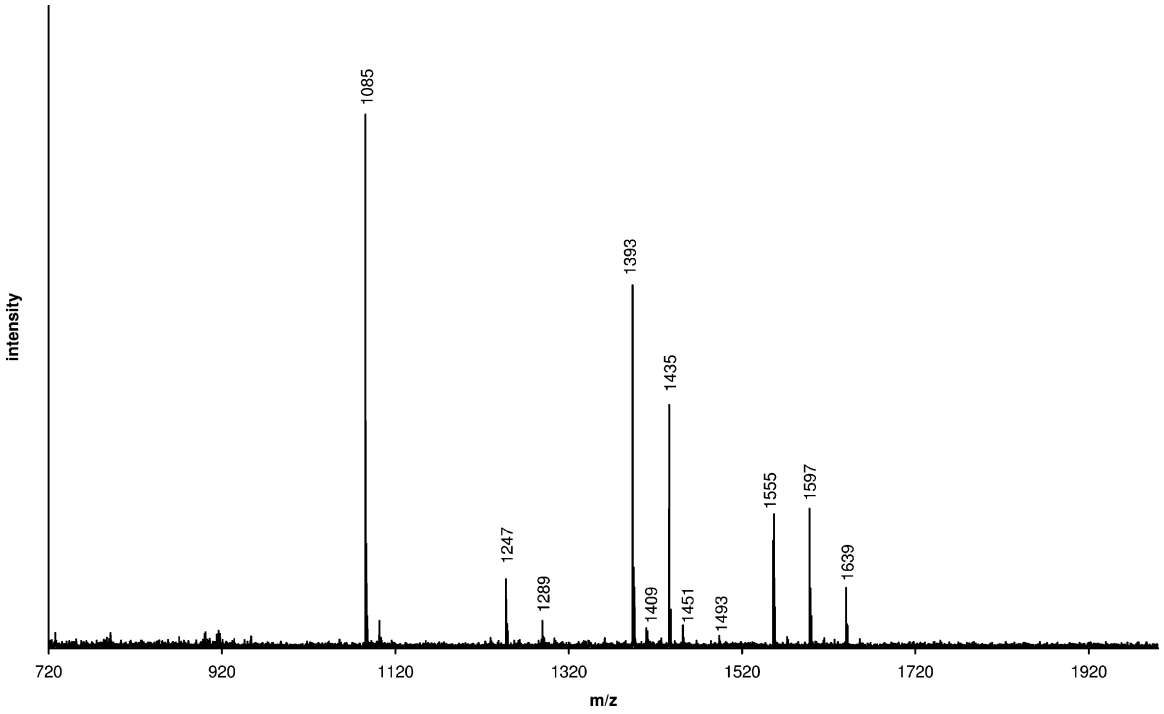

Fig. 5. MALDI–TOF mass spectrum of xyloglucan oligomers obtained after XEG treatment of black currant AIS (only sodium adducts are labelled).

attributed to XLXG or XXLG ($m/z = 1247$). However, a distinction of the two oligomers with the same mass is not possible by MALDI–TOF–MS.

Comparing the mass spectrum of xyloglucan oligosaccharides derived from CASS (Fig. 4) to the mass spectrum of the ones derived from AIS (Fig. 5), six additional peaks were present in the mass spectrum of AIS. These oligomers carried one ($m/z = 1289$, $m/z = 1435$, $m/z = 1451$, $m/z = 1597$) or two ($m/z = 1493$, $m/z = 1639$) acetyl groups. Only the galactose containing oligosaccharides were acetylated. The oligosaccharides that carry two galactose residues (XLLG, XLFG) contained even two acetyl groups. Acetylation was reported to occur on the galactose residue of xyloglucan [22,34]. Other possible binding sites for acetyl groups are arabinose units or O-6 of a glucose unit in XXGG-type xyloglucans [37], but those kinds of oligomers were not present in black currant xyloglucan.

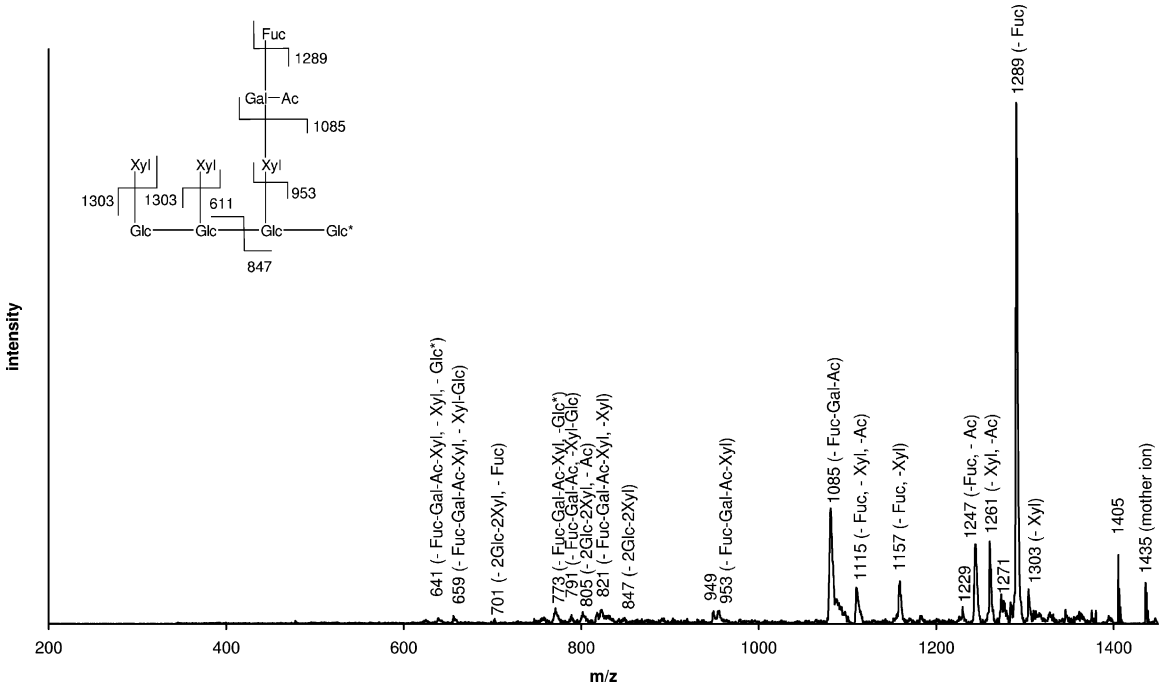

Fig. 6. Post source decay (PSD) spectrum of acetylated XXFG ($m/z = 1435$, structure is shown in the left top corner).

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MALDI–TOF–MS is a fast technique for oligosaccharide profiling and for demonstrating the presence of acetylated oligomers. However, oligomers having the same molecular mass are not distinguished. In time consumption and handling MALDI–TOF–MS outclasses ESI–MS, but structural characterisation of oligomers including localising of acetyl groups is not possible.

3.4. MALDI–TOF–MS–PSD

To identify the positions of the different side chain and of the acetylated residues in the xyloglucan building blocks, post source decay (PSD) provides more structural information [4].

From black currant AIS a xyloglucan oligomer with a mass of an acetylated XXFG (sodium adduct $m/z = 1435$) was released as shown by MALDI–TOF–MS (Fig. 5). The PSD-spectrum and the structure of this oligomer are shown in Fig. 6. The asterisk (*) marks the reducing end of this oligomer, which is cleaved from the oligomer always in the hydrated form ($m = 180$ Da). The peak with the highest intensity corresponded to the mass of the oligomer without the fucose unit ($m/z = 1289$). This excluded already acetylation of the fucose residue in XXFG, although a fragment of XXFG without fucose and acetyl was shown ($m/z = 1247$). Thus, the acetyl group was able to leave the molecule independently. The second peak in intensity ($m/z = 1085$) corresponded to the oligomer without fucose and an acetylated hexose. The only non-reducing hexose that can leave the oligomer after the fucose is removed is the galactose residue. Therefore, this mass to charge ratio of 1085 indicated that the galactose was acetylated, although a fragment without a pentose and an acetyl group ($m/z = 1261$) that would indicate acetylation of a xylose was shown in lower intensity. The fragments with $m/z = 805$ and $m/z = 847$ corresponded to the FG* and acetylated FG*, respectively, and confirmed the position of the F side chain next to the reducing end. In addition, the fragment with $m/z = 847$ showed that the acetyl group was present on the FG* fragment. Since acetylation on the reducing glucose would inhibit enzymatic cleavage of xyloglucan at this position [35], acetylation of the galactose unit was most probable, but could not be proven by PSD.

PSD spectra of all acetylated oligomers indicated acetylation of galactose, as known for xyloglucans from sycamore cells [22,34]. Double acetylation of one residue as reported for extracellular xyloglucan of sycamore cells [22] was not seen. Furthermore, the $m/z = 1247$ peak was identified as a mixture of XXLG and XLXG and the structure of XLFG was confirmed with PSD, as well.

In xyloglucan analysis PSD can be used for identification of the side chain position of xyloglucan oligomers. However, definitive confirmation of the position of the acetyl group is difficult. The background noise is high and the accuracy of mass to charge ratios is not always optimal. Therefore, ESI–MS^n of the oligomers was performed to confirm the results of PSD, to prove the position of the acetyl groups, and to compare the two techniques.

3.5. ESI–MS^n

Alternatively to PSD, electrospray ionisation mass spectrometry (ESI–MS) with ion trap is an opportunity to fragment selected ions and to get an insight in the structure of xyloglucan oligomers. ESI–MS^n is applicable for both, mixtures and fractions derived from the analytical HPAEC after SPE. Mild

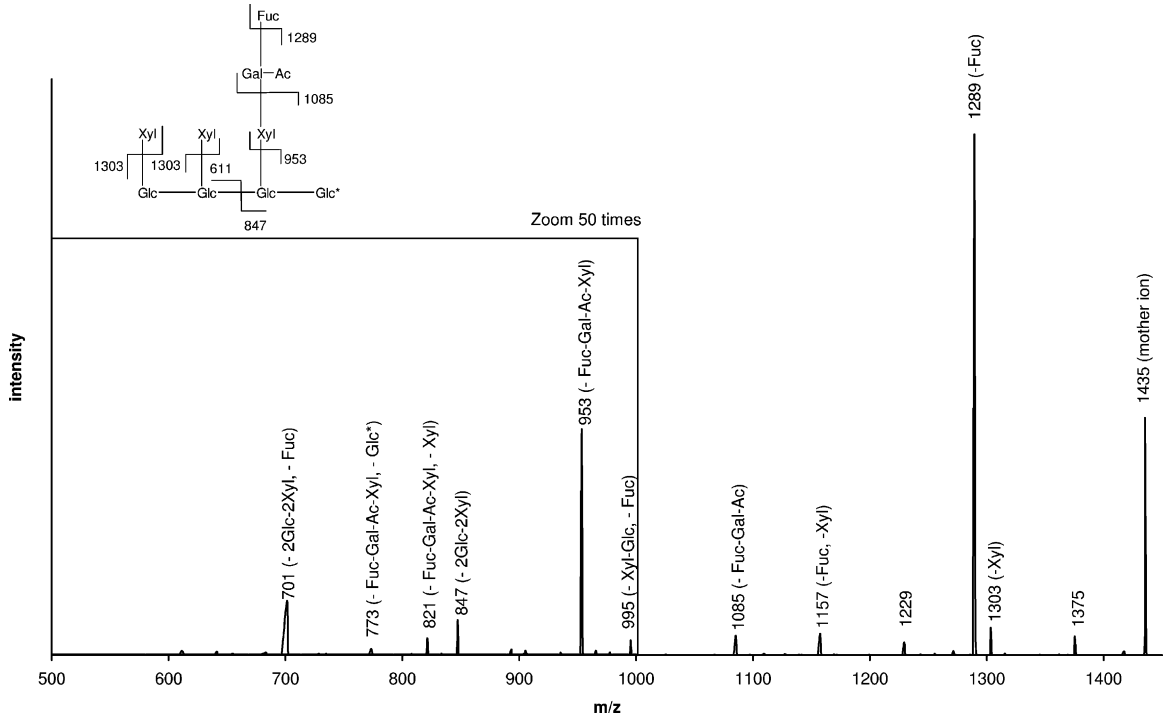

Fig. 7. ESI–MS^2-spectrum of actylated XXFG oligomer (structure is shown in the left top corner).

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fragmentation conditions were used to obtain glycosidic cleavages rather than cross ring cleavages.

The ESI–MS^2 spectrum of acetylated XXFG ($m/z = 1435$) showed less back ground noise and better resolved peaks (Fig. 7) compared to the PSD spectrum of the same oligomer (Fig. 6). The sensitivity was much higher, so that a 50 times zoom in the lower m/z region below $m/z = 1000$ showed still very well resolved peaks and hardly any background noise.

When the mother ion of acetylated XXFG ($m/z = 1435$) was fragmented, the most apparent peak corresponded to the oligomer without fucose as already seen with PSD. Other peaks present proved that the acetyl group was present at the galactose unit. Only when the galactose unit was cleaved off the acetyl group was removed, as well. When a deoxy hexose and a hexose left the oligomer, the acetyl group left as well ($m/z = 1085$). However, when a deoxy hexose was cleaved together with a pentose, the acetyl group remained at the main ions ($m/z = 1157$). With ESI–MS^2 the acetyl group did not leave the oligomer independently from the sugar residue, as it was the case for PSD. The removal of fucose and xylose at the same time (fragment $m/z = 1157$) can only be explained by a double cleavage in the xyloglucan oligomer: The fragment $m/z = 847$ in the MS^2 spectrum of $m/z = 1435$ together with the same fragment without fucose ($m/z = 701$) in the MS^3 spectrum of the defucosilated oligomer ($m/z = 1289$) showed fucosilation of this fragment. The MS^4 spectrum of the $m/z = 701$ fragment showed the removal of acetylated galactose (remaining fragments $m/z = 497$), but no xylose (theoretical $m/z = 569$). Therefore, fucose can only be attached to galactose, as reported before [38,39], and not to xylose.

With ESI–MS^2 it was possible to identify the galactose residue of xyloglucan oligomers as carrier of the acetyl groups. Double acetylation on one galactose unit as seen in extracellular xyloglucan of sycamore cells [22] was not found in black currant xyloglucan. The double acetylated oligomer of XLFG ($m/z = 1639$) carried the acetyl groups on two different galactose units. Furthermore, the structures of the xyloglucan oligomers XXXG, XXFG, XLFG, XLLG, and a mixture of XLXG and XXLG were confirmed.

ESI–MS^2 with ion trap is a fast and sensitive method for the determination of oligosaccharide structures. It is more sensitive than PSD and for oligomers of which MS^2 is ambiguous MS^n can be performed, depending on the concentration to MS^6 or MS^7. Especially the low background noise, which allows identification and further fragmentation of m/z peaks with a sensitive device even in 50-times zoom, makes ESI–MS^n superior of MALDI–TOF–MS–PSD.

3.6. RP–HPLC

An alternative to HPAEC is reversed phase HPLC (RP–HPLC). Xyloglucan oligosaccharides derived from CASS can be separated. The advantage of RP–HPLC is the online connection to a mass spectrometer parallel to an evaporate light scattering detector (ELSD). A desalting procedure is not necessary.

The chromatogram of xyloglucan oligosaccharides derived from CASS is shown in Fig. 8. Four xyloglucan oligomers were identified by online ESI–MS and ESI–MS^2. These oligomers were also identified by HPAEC (Fig. 3). RP–HPLC separated the alpha and beta anomers of each oligomers [24] so that the chromatogram showed two peaks with the same mass. The area percentage differed from the area percentage of the HPAEC, although the general tendency with XLFG being the most appar-

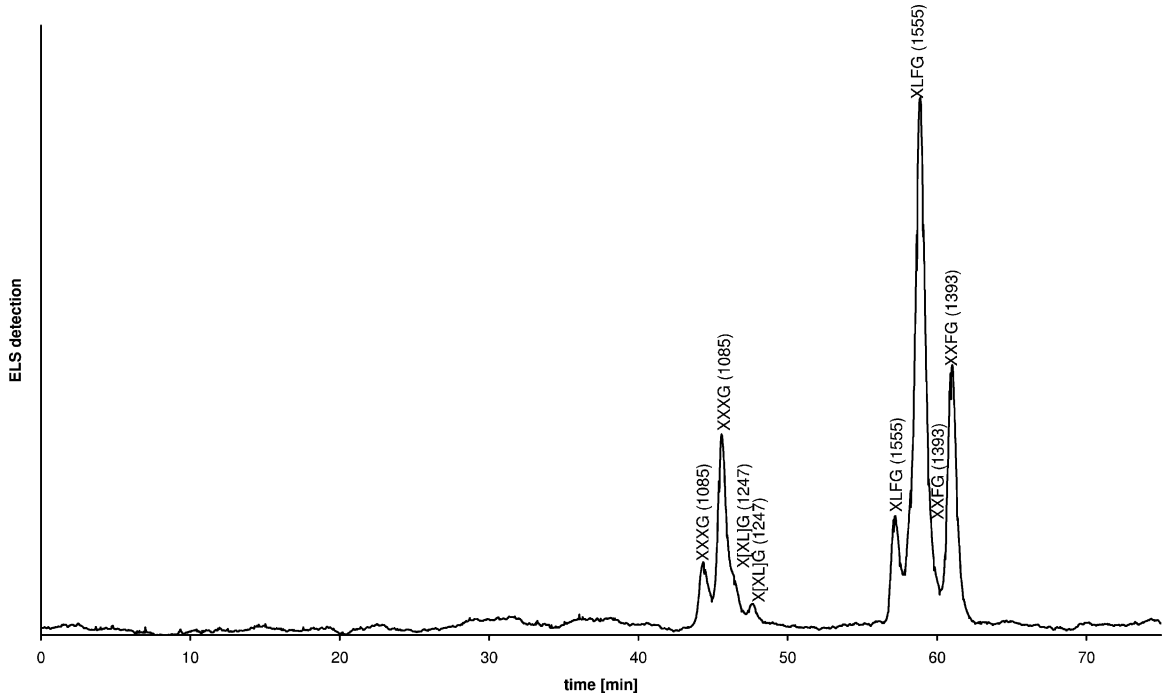

Fig. 8. RP–HPLC elution pattern of xyloglucan oligosaccharides derived from CASS.

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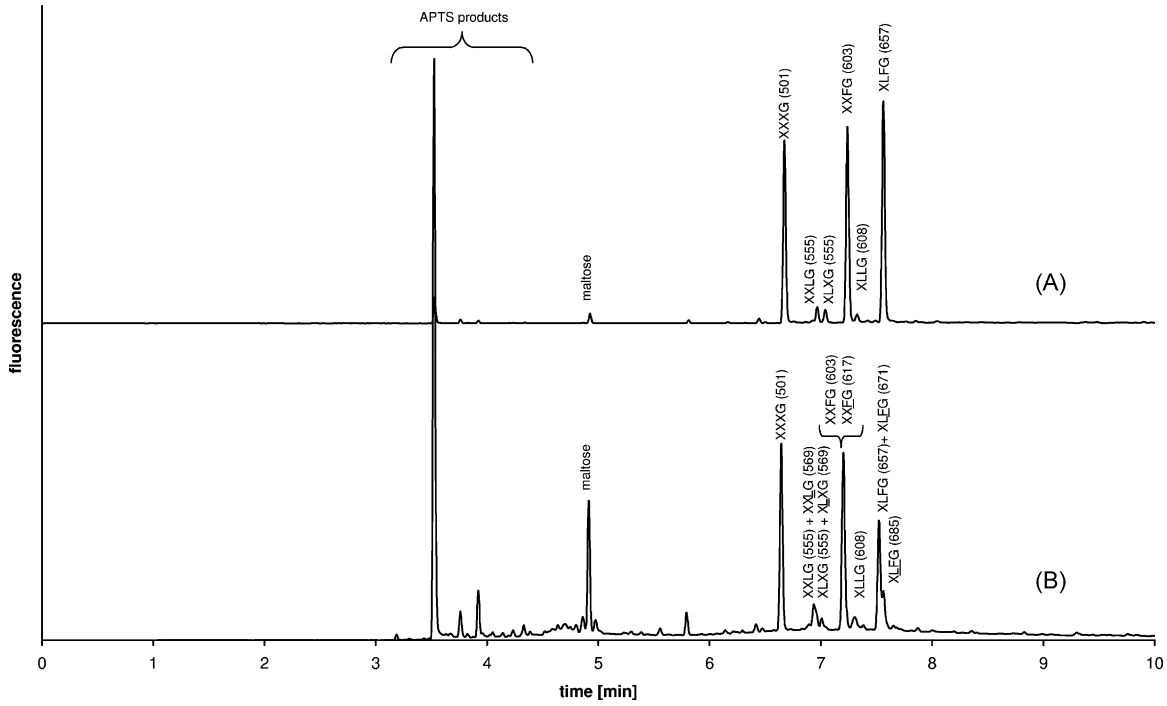

Fig. 9. Electropherograms of APTS labelled xyloglucan oligosaccharides derived from black currant CASS (A) and AIS (B). In brackets the *m/z* values of the triple charged, labelled oligomers are shown. Acetylation is indicated by _ and maltose was used as an internal standard.

ent oligosaccharide in xyloglucan oligosaccharides derived from CASS was the same for both techniques. While the differences between the areas of the XXXG, XXFG, and XLFG peaks were only small compared to those on HPAEC, the area of XLFG (57%) was more than double of the other two main oligosaccharides XXXG (21%) and XXFG (20%) with RP–HPLC. This may be partly due to different response factors of the ELSD to the HPAEC–PAD [36]. The oligomer XLLG was not detected by this RP–HPLC–MS and the oligomers XXLG and XLXG were not separated.

RP–HPLC is an alternative to HPAEC for separation and online identification (ESI–MS^n) of different xyloglucan oligomers. However, our RP–HPLC system did not allow a faster or better separation than HPAEC. Not all oligomers were detected and separation of alpha and beta anomers makes the chromatograms more complicated. When an HPAEC system is available, it preferably should be used.

3.7. CE–LIFD and CE–MS

Recently capillary electrophoresis (CE) became of interest in the carbohydrate analysis [40]. Advantages of CE are short measuring times and good resolution of the different compounds, while hardly any solvents are needed. However, labelling of neutral oligosaccharides with for example APTS is necessary [40]. With APTS three charges are introduced, which allow the neutral oligomers to migrate under electrophoretic conditions. Furthermore, APTS is fluorescent and allows laser induced fluorescence detection (LIFD) of the labelled sugars. By using an internal standard, quantification of the different oligomers is possible.

The electropherograms of APTS labelled xyloglucan oligosaccharides derived from black currant CASS and AIS are shown in Fig. 9. Components migrating to the detector between 3 and 4 min were degradation products of the label (APTS). After 4.9 min the internal standard maltose and between 6 and 8 min the xyloglucan oligomer peaks were detected. The electropherogram of xyloglucan oligosaccharides derived from AIS differed from the one derived from CASS in having a double peak instead of a single peak at the last oligomer peak. Identification of the peaks and, therefore, the electrophoretic mobility of the different labelled oligomers was not possible by LIFD.

When the CE was, however, coupled online to an ESI–MS^n detector, the labelled oligomers were identified by their mass to charge ratios. The conditions for CE–MS were modified to obtain the same order of migration by using a longer capillary that had no polyvinyl alcohol coating compared to CE–LIFD. To suppress the stronger electro osmotic flow in the capillary, the buffer pH was lowered. Due to the introduction of three negative charges with the APTS-label, the oligomers were mainly detected as triple charged ions. The mass to charge ratios are shown in the CE–MS detected electropherogram (Fig. 10), which showed only a slight decrease in resolution compared to CE–LIFD. The time of the measurement was nearly doubled due to a longer capillary used to couple CE and ESI–MS. We even managed to obtain high quality MS^2 spectra of the CE separated, APTS labelled oligomers (Fig. 10).

The smallest oligomers had the highest electrophoretic mobility and were detected first, while the largest oligomers were detected last due their lower electrophoretic mobility. The area percentages were comparable to HPAEC, but in contrast to HPAEC the area percentage determined by CE–LIFD is equal to

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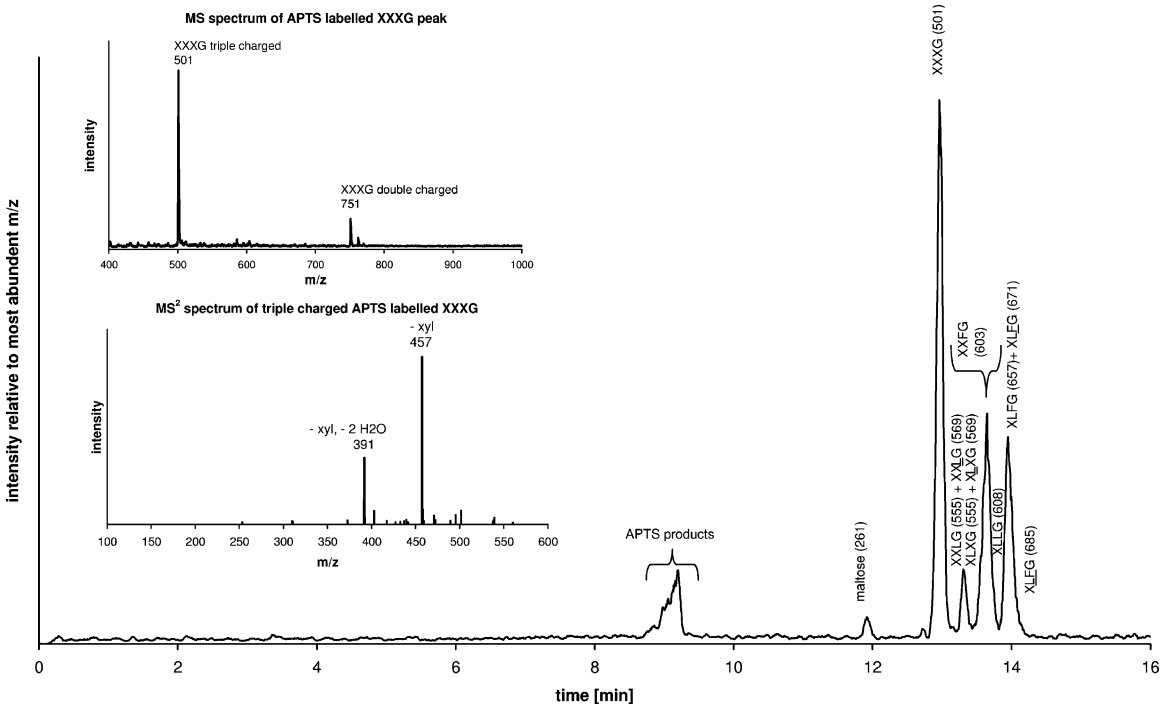

Fig. 10. Electropherograms of APTS labelled xyloglucan oligosaccharides derived from black currant AIS detected by ESI–MS.

the molar percentage due to the fluorescent label on the reducing end. In the electropherogram from CASS the oligomer XLFG was the main oligomer (35%), followed by XXFG (31%) and XXXG (28%). XLXG (2%), XXLG (2%), and XLLG (1%) were minor oligomers. XLXG and XXLG were identified by their MS^2 spectra. Similar results were obtained by using HPAEC, but RP–HPLC–ELSD analysis shows a higher area percentage of XLFG. Acetylated oligomers present in the AIS digest were detected by CE–MS. Monoacetylated oligomers were not separated from the oligomers without an acetyl group, but XLFG carrying two acetyl groups was. Thus, a double peak was detected in the electropherogram of xyloglucan oligomers derived from AIS, where a single peak of XLFG was detected in the electropherogram of xyloglucan oligomers derived from CASS. With CE the different compositions of AIS xyloglucan compared to CASS xyloglucan was seen. In AIS xyloglucan XXFG (32%) was the main xyloglucan oligosaccharides followed by XXXG (30%) and XLFG (25%). These results differed from the results of the HPAEC determination where XXXG was the main oligomer. The differences were, however, small and can be explained by different response factors on HPAEC–PAD [36].

Xyloglucan oligomers of tamarind and cotton showed the same order of electrophoretic mobility under similar CE–LIFD conditions [20]. The authors do not give measurement times, but most likely our results are comparable due to similar systems used.

CE–LIFD is a time and solvent saving technique. Resolution of the oligomer separation is better and faster compared to HPAEC and RP–HPLC. The possibility to connect CE online to an ESI–MS^n allows the identification of known and unknown xyloglucan oligomers and, therefore, their electrophoretic mobility. CE also separates double acetylated oligomers from single or non-acetylated ones. These advantages compensate the necessity to label the oligomers.

4. Conclusions

We presented different techniques to analyse the structure of xyloglucan in black currants. All three separation techniques (HPAEC, RP–HPLC, and CE) show different elution orders of xyloglucan oligosaccharides. With each of these techniques it is possible to characterise xyloglucans by their oligomers obtained after enzyme degradation. HPAEC and CE showed similar separation profiles, while RP–HPLC was not able to separate all oligomers. MALDI–TOF–MS and ESI–MS^n can be used either instead, or additionally, or coupled off line to HPAEC or online to RP–HPLC or CE–MS, as newly presented in this paper. Combination of different techniques allows oligosaccharides profiling and structural characterisation of unknown oligosaccharides leading to an unambiguous xyloglucan structure. If it is necessary to limit the number of techniques, CE–LIFD is the fastest way to quantify xyloglucan oligomers. In case of unknown oligomers present, identification by online ESI–MS^n is possible. MALDI–TOF–MS can be used for fast oligosaccharide profiling, because many samples can be analysed in a short time. For structural characterisation ESI–MS^n on a sensitive device outclasses PSD.

Black currant xyloglucan is of XXXG-type and contains galactose (L) and fucose-galactose (F) side chains in three major (XXXG, XXFG, and XLFG) and four minor building blocks (XXG, XLXG, XXLG, and XLLG). Black currant xyloglucans are the first xyloglucan analysed in the *Grossulariacease* family and the *Saxifragales* order.

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