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Chromatography based profiling of feruloylated arabinans and galactans

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ABSTRACT

Profiling of pectic arabinans and galactans by analysis of the released oligosaccharides after backbone cleavage provides information on the complexity of the polymer structure. In plants of the family Amaranthaceae, arabinan and galactan substitution with ferulates extends the polysaccharide complexity, changing its chemical properties. Knowledge of the ferulate environment is crucial to understand structure-function-relationships of feruloylated pectins. Here, we present an approach to separate enzymatically generated feruloylated and non-feruloylated arabino- and galactooligosaccharides, followed by deesterification and semiquantitative analysis by HPAEC-PAD using previously reported relative response factors. Application of this approach to sugar beet pectins and insoluble and soluble dietary fiber preparations of amaranth and quinoa suggests that ferulates are preferably incorporated into more complex structures, as nicely demonstrated for feruloylated galactans. Also, ferulate substitution appears to negatively affect enzymatic cleavage by using *endo*-enzymes. As a consequence, we were able to tentatively identify new feruloylated tri- and tetrasaccharides of galactans isolated from sugar beet pectins.

1. Introduction

Together with cellulose and hemicelluloses, pectins contribute to the polysaccharides of the cell wall. Pectins make up most of the middle lamella and are therefore found in all plant tissues. The structures of primary cell walls differ depending on taxonomy. Commelinid monocotyledonous plant cell walls are built mainly of cellulose and (arabino) xylans, whereas pectins make up only a small portion. In contrast, dicotyledonous plants and non-commelinid monocotyledonous plants contain pectins and xyloglucans as major constituents of their primary cell walls. Although they are potentially involved in the formation of the architecture of secondary cell walls [1,2], pectins play only a minor role in secondary and tertiary cell walls in quantitative terms. Due to their unique properties, pectins are widely used in the food industry as gelling, thickening, stabilizing, or emulsifying agents [3,4]. Pectins are mainly composed of homogalacturonan and rhamnogalacturonan I (RG I). Homogalacturonan represents an α-1,4-linked polymer of D-galacturonic acid units. In contrast, RG I is composed of alternating α-1,4-linked D-galacturonic acid and α-1,2-linked L-rhamnose units. Further complexity is due to partial substitution of rhamnose units at position *O*-4 with arabinans and galactans as neutral sidechains (Fig. S1) [5,6]. Arabinans are generally described as α-1,5-linked L-arabinofuranose based polymers, with the arabinose units being partially substituted with L-arabinofuranose units at position *O*-2 and/or *O*-3 [7]. Additional structural units have been described depending on taxonomy [8–10]. Differently, galactans are mainly linear polymers composed of β-1,4-linked D-galactopyranose units, which may incorporate arabinose units at positions *O*-3 or *O*-6 or even within the backbone [6,10–12]. Arabinans and galactans in plants of the family Amaranthaceae such as sugar beet (*Beta vulgaris* L. var. *vulgaris*), amaranth (*Amaranthus* sp.), and quinoa (*C. quinoa* WILLD.) are partially acylated with *trans*-ferulic acid at position *O*-2 of arabinose units or position *O*-6 of galactose units [13–16]. Ferulate oxidation results in the formation of oligoferulates, leading to intra- and/or intermolecular crosslinks [17]. This oxidation can also be performed *in vitro* in order to generate covalent hydrogels with unique properties [18,19]. Knowledge of the arabinan and galactan structures is crucial for understanding structure-function-relationships of these polymers in the plant as well as of the isolated pectins in hydrogels and other applications. For example, it was previously demonstrated that arabinans affect the emulsifying properties of sugar beet pectins [3,20]. Therefore, methods that allow for a fast structural profiling of these pectic neutral side chains were established. Documentation of released oligosaccharides after enzymatic digestion is among the most accurate tools to describe the composition of arabinans and galactans because chemical modifications, e.g. through acidic treatments, can be excluded [21]. For example, the use of endo-α-1,

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4-arabinanase catalyzes the cleavage of linear α-1,4-coupled arabinans, resulting in the release of several branched arabino oligosaccharides. In contrast, β-1,4-galactanase cleaves linear β-1,4-linked galactoses in galactans. Analysis of the liberated oligosaccharides is thereby mostly carried out by nuclear magnetic resonance (NMR) or high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) [21,22]. Wefers and Bunzel [23] made this enzymatic approach easier to handle for laboratories by using a semi-quantitative profiling approach for the released arabino- and galacto-oligosaccharides that is based on relative response factors. The examined arabino- and galacto-oligosaccharides are summarized in Figs. 1 and 2. However, due to the (comparatively) low abundance of feruloylated oligosaccharides and (partial) deesterification under alkaline conditions in HPAEC-PAD, information on feruloylation is lost. Access to feruloylated oligosaccharides is mostly gained by elaborate, (semi)preparative fractionation using Amberlite and Sephadex LH-20 materials [13,24,25]. However, application of these separation media in an analytical profiling approach appears to be unsuitable, where less time-consuming sample preparation steps are necessary. Therefore, we developed a simple separation of feruloylated (FAOS) and non-feruloylated oligosaccharides (NFAOS) after enzymatic cleavage of arabinans and galactans, followed by deesterification prior to HPAEC-PAD analysis to effectively describe the molecular environment of ferulates in pectins from amaranthaceous plants in a semiquantitative approach.

2. Results and discussion

2.1. Method development

FAOS and NFAOS are released by enzymatic degradation of arabinans and galactans. Optimum conditions for the enzymatic degradation of (non-feruloylated) arabinans and galactans by using *endo*-arabinanase and *endo*-galactanase were examined previously by Wefers and Bunzel [23]. Here, these conditions were initially used to degrade Pct2 and Pct4. Pct2 is a sugar beet pectin extracted under mild acidic conditions and with high portions of arabinans, which was chosen as reference material for the enzymatic release of (feruloylated and non-feruloylated) arabino-oligosaccharides. Contrarily, Pct4 depicts a sugar beet pectins with a larger proportion of galactans due to harsher acidic extraction conditions. Therefore, Pct 4 was chosen to study the enzymatic release of (feruloylated and non-feruloylated) galacto-oligosaccharides.

Because both FAOS and NFAOS are released by enzymatic digestion, FAOS and NFAOS need to be separated, e.g. by liquid chromatography (LC). FAOS are known to interact with reversed phase (RP) stationary phases such as C18- and phenylhexyl-based materials [26], whereas pure carbohydrates are supposed to be little retarded by RP stationary phases. Fig. 3 shows the optimized evaporative light scattering (ELSD)- and UV (325 nm)-detected chromatograms of hydrolysates that were obtained after enzymatic treatment of Pct2 and Pct4 on a semi-preparative phenylhexyl column. A distinct separation in two fractions was observed, with the first fraction (10.0–15.0 min) inducing a large ELSD- and a small UV-signal and the second fraction (29.5–31.0 min) being characterized by a small ELSD- but large UV-signal. In order to characterize the behavior of pure carbohydrates and phenolics under these conditions, maltose and several ferulates were analyzed. Maltose co-eluted with fraction 1, and fraction 2 showed a similar elution behavior as *trans*-ferulic acid, 8-*O*-4- and 8-8-non-cyclic-diferulic acids, and 8-*O*-4/8-8-triferulic acid (data not shown). Thus, fraction 1 appears to contain NFAOS and fraction 2 FAOS.

To further test this hypothesis, both fractions were collected and analyzed by LC with mass spectrometric (MS) detection. Direct injection of fraction 1 of the *endo*-arabinanase hydrolysate of Pct2 into the ESI-source revealed the existence of pentose oligosaccharides with polymerization degrees up to 11 (Fig. S2). To confirm the existence of NFAOS in this fraction, fraction 1 after *endo*-arabinanase digestion was also analyzed after separation on a porous graphitized carbon (PGC) column coupled to an MS, which has been used for the analysis of NFAOS in the past [10] and which is suitable for the detection of oligosaccharides in low concentrations. Typical signals of pentose based oligosaccharides with polymerization degrees up to seven were found, confirming the existence of NFAOS in fraction 1.

Because FAOS were supposed to irreversibly bind to a PGC column, fraction 1 was analyzed on an RP column using LC-UVD/MS, too. However, following separation on a C18 column that has been previously used for the separation of FAOS liberated from arabinoxylans [27], fraction 1 showed no UV-active signals and no signals of known m/z ratios. This suggests that fraction 1 does not contain FAOS.

Direct injection of fraction 2 of the *endo*-arabinanase hydrolysate into the ESI-source of the MS showed the existence of pentose containing oligosaccharides with polymerization degrees up to 11, each carrying a single *trans*-ferulate unit ($[M+Na]^+$ = up to 1669), whereas ions that represent NFAOS were not existent (Fig. S2). Analysis of fraction 2 by using PGC-LC-MS did not detect any traces of NFAOS, too. In contrast, FAOS with polymerization degrees up to 7 were identified by LC-UVD/MS after separation of fraction 2 on a C18 column. It is noteworthy that signals of oligosaccharides which are acylated with diferulic acids (or even higher ferulate oligomers) were not identified probably due to low concentrations.

Basically the same behavior was shown for fractions 1 and 2 of the *endo*-galactanase hydrolysates of Pct4 (Fig. S3). However, the degree of polymerization of the identified oligosaccharides was smaller (up to 4). Thus, the developed method is capable of separating FAOS and NFAOS within a single chromatographic step.

To identify and semiquantitatively analyze arabino- and galacto-oligosaccharides as NFAOS in fraction 1, HPAEC-PAD based analysis and relative response factors as previously published were applied [23]. In order to apply these relative response factors to the feruloylated arabino- and galacto-oligosaccharides in fraction 2, too, it needs to be ensured that - either during the alkaline conditions of HPAEC or before injection - ferulic acid is quantitatively removed from the FAOS. HPAEC conditions were shown to insufficiently saponify FAOS as indicated by tailing and low intensity peaks (Fig. S4). Therefore, an enzymatic degradation of ferulate esters using a mixture of two feruloyl esterases prior to HPAEC-PAD analysis was necessary. Peaks in the HPAEC-PAD-chromatograms of the deesterified oligosaccharides were proven to be sharp and more intense if compared to the FAOS without prior deesterification (Fig. S4). It is noteworthy that salts that were present in the esterase mixtures used lead to retention time shifts (2 min) in the middle segment of the chromatograms. Complete degradation was demonstrated by RP(C18)-LC-UVD/MS analysis of feruloyl esterase digested fraction 2. Also, when this enzyme digested fraction 2 was analyzed by PGC-LC-MS the non-feruloylated oligosaccharides were nicely detected. Because the arabino- and galacto-oligosaccharides that were liberated from fraction 2 by using feruloyl esterase were identical to the non-feruloylated ones in fraction 1, they can be analyzed by HPAEC-PAD using the same conditions as described for the NFAOS if the retention time shifts are taken into account. Thus, our previously described response factors can also be used for the arabino- and galacto-oligosaccharides in fraction 2. Since the feruloyl esterase used is only capable of catalyzing the release of monomeric ferulic acid, any diferuloylated oligosaccharides that may be present are probably still part of the mixture. Presumably, they are contained in comparably low concentrations; thus, they are not separately considered in this approach.

2.2. Preparation and characterization of sample materials

Samples were selected according to their botanical origin including plants of the family Amaranthaceae such as sugar beet, amaranth, and quinoa that are known for feruloylated arabinan and galactan structures.

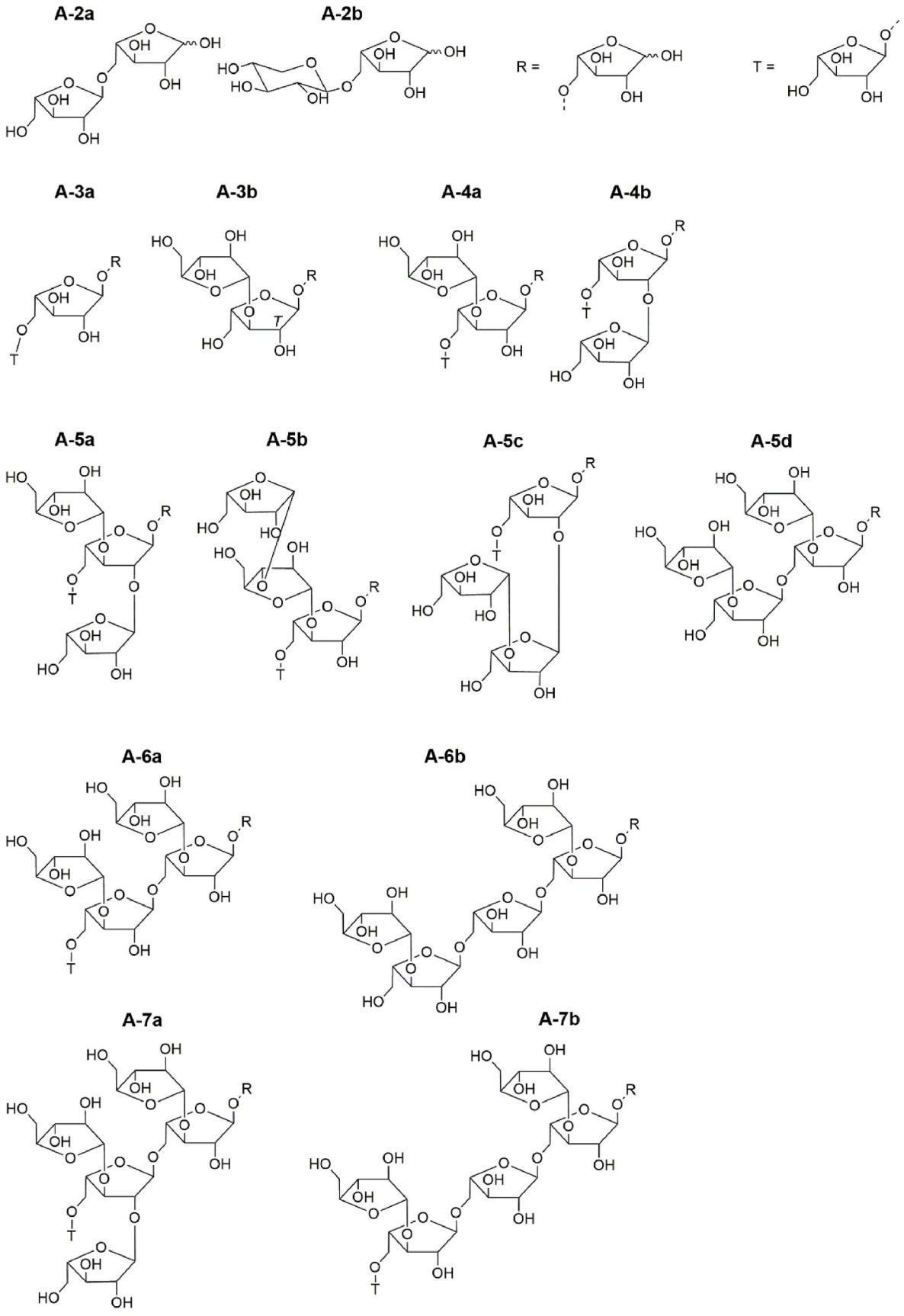

Fig. 1. Arabino-oligosaccharides liberated after enzymatic cleavage with *endo*-arabinanase as described by Wefers and Bunzel [23].

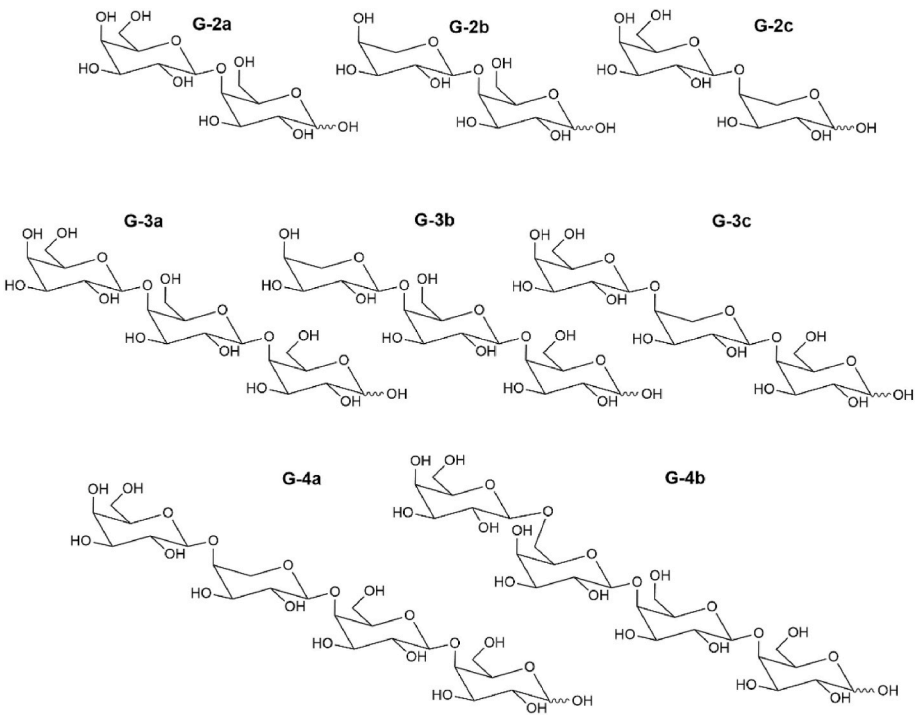

Fig. 2. Galacto-oligosaccharides liberated after enzymatic cleavage with *endo*-galactanase as described by Wefers and Bunzel [23].

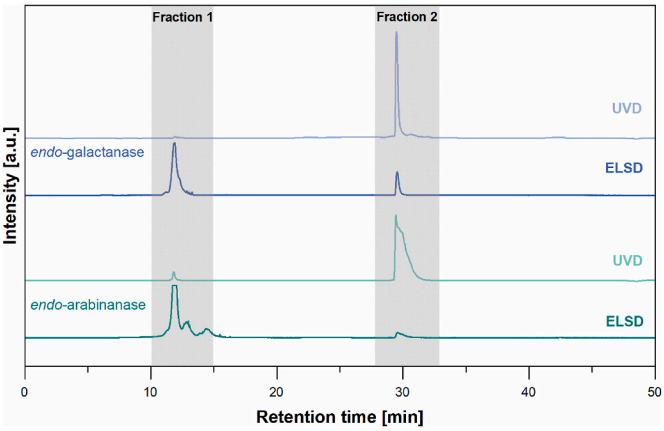

Fig. 3. Evaporative light scattering (ELSD) and UV (325 nm)-detected chromatograms of hydrolysates after *endo*-galactanase digestion of Pct4 (top) and *endo*-arabinanase digestion of Pct2 (bottom) after separation on a semi-preparative phenylhexyl column. The grey areas depict the two collected fractions, which were further analyzed.

Water soluble and insoluble fractions (soluble dietary fiber (SDF) or insoluble dietary fiber (IDF)) were prepared from amaranth and quinoa seeds. Structurally different sugar beet pectins were obtained by extracting pectins under varying conditions as described by Junker et al. [18]. Extraction yields and monosaccharide compositions of these pectin fractions (Pct) are given in Table 1. In contrast to Pct1-5, Pct6 has not yet been described, but its monosaccharide composition compares to Pct2, indicating a large portion of arabinans and galactans.

Both amaranth and quinoa IDF polysaccharides contain large amounts of glucose, which is mainly derived from cellulose. Due to high contents of galacturonic acid, rhamnose, arabinose, and galactose, pectins appear to represent a large portion of amaranth and quinoa IDF and SDF.

Table 2 shows the results of the arabinan and galactan profiling approach without differentiation between NFAOS and FAOS, thus, representing the data that are obtained by using our previously published profiling approach [23]. IDF fractions were subjected to an autoclave treatment prior to the enzymatic degradation to loosen cell wall structures and thus, to facilitate the subsequent enzymatic cleavage [14].

The structures of the identified oligosaccharides are shown in Figs. 1 and 2. Pct of sugar beet show large portions of complex arabinans with branches in position *O*-3 (A-3b, A-4a, A-6a, A-7b). Additionally, substitution at position *O*-2 (A-4b) as well as double substitution of arabinofuranose units at positions *O*-2 and *O*-3 (A-5a, A-7a) occur, however, in minor portions. With increasing extraction time at pH 2 (Pct2-4), arabinans become debranched as indicated by the reduction of portions of complex compounds such as A-4a (16.8 mol% to 9.0 mol%) and A-7b (11.5 mol% to 0 mol%). Profiling data that indicate debranching are supported by methylation analysis data (Table S1) as substituted structures (1,3,5- and 1,2,3,5-substituted arabinofuranose units) decrease with increasing extraction time. However, relative portions of A-3b, an additional 1,3,5-linked compound, increase with increasing extraction time at pH 2. In the past, A-3b has been described as a product of an arabinofuranosidase side activity of the arabinanase as it lacks a terminal arabinofuranose unit if compared to compound A-4a. The large content of up to 46 mol% in Pct4 may also indicate an arabinan modification under acidic conditions prior to the enzymatic cleavage. However, as methylation analysis does not suggest larger amounts of this structural unit in Pct4, the large portion of A-3b in this particular Pct cannot satisfyingly be explained. Sonication has no distinct effect on arabinan structures (Pct2 vs. Pct6). IDF of amaranth and quinoa also contain complex arabinans although the portions of larger (and thus more complex) oligosaccharides are lower compared to sugar beet arabinans. It is noteworthy that amaranth SDF show large amounts of A-3a, a linear trimer of 1,5-linked arabinose units. It is known to be an intermediate structure as it can be further digested to A-2a and arabinose and may thus be used as an indicator for an incomplete enzymatic hydrolysis of arabinans [23].

By using the galactan profiling approach without preseparation of NFAOS and FAOS it was demonstrated that sugar beet pectin galactans are mainly linear with considerable amounts of terminal

Table 1
Extraction yields (g/100g) and monosaccharide compositions (mol%) after methanolysis and trifluoroacetic acid hydrolysis of six extracted sugar beet pectin fractions and soluble dietary fiber (SDF) preparations of amaranth and quinoa seeds and, after sulfuric acid hydrolysis, of insoluble dietary fiber (IDF) preparations of amaranth and quinoa seeds. US, ultrasonic; n.q., detected but not quantifiable; SD, standard deviation.

	Pectin fraction						Amaranth		Quinoa	
	1[a]	2[a]	3[a]	4[a]	5[a]	6	IDF	SDF	IDF	SDF
	Autoclave	1 h, pH 2	4 h, pH 2	16 h, pH 2	4 h, pH 3	30 min US, 1h, pH 2				
Yield[b]	15.0 ± 0.2	14.1 ± 0.4	17.3 ± 0.0	20.6 ± 0.7	10.1 ± 0.9	12.2 ± 0.2	10.5 ± 0.3	3.3 ± 0.2	4.8 ± 0.04	4.4 ± 0.1
					Monosaccharide composition[c]					
Fucose	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	0.9 ± 0.02	–	0.7 ± 0.04	–
Rhamnose	6.1 ± 0.3	7.1 ± 0.2	9.8 ± 0.3	14.1 ± 0.1	5.4 ± 0.2	7.5 ± 0.3	3.3 ± 0.1	5.6 ± 0.1	1.9 ± 0.06	7.0 ± 0.1
Arabinose	47.9 ± 4.4	42.5 ± 0.1	20.5 ± 0.4	6.8 ± 0.1	46.4 ± 1.3	41.6 ± 1.7	21.4 ± 0.3	33.7 ± 0.8	18.0 ± 1.1	21.2 ± 0.05
Galactose	16.8 ± 1.8	17.7 ± 0.4	20.9 ± 0.2	24.9 ± 0.3	15.1 ± 0.5	16.2 ± 0.5	4.7 ± 0.1	11.5 ± 0.1	3.4 ± 0.1	7.5 ± 0.2
Glucose	3.2 ± 0.3	2.8 ± 0.1	3.5 ± 0.2	4.0 ± 0.04	3.5 ± 0.1	1.4 ± 02	40.8 ± 0.4	3.6 ± 1.2	60.7 ± 2.0	2.6 ± 1.4
Xylose	–	–	–	–	–	–	7.7 ± 0.2	12.7 ± 1.0	5.9 ± 0.2	6.1 ± 0.2
Mannose	–	–	–	–	–	–	1.0 ± 0.1	18.5 ± 0.5	2.8 ± 0.2	14.7 ± 0.5
Galacturonic acid	24.9 ± 5.5	28.5 ± 0.5	43.4 ± 0.8	47.8 ± 0.3	28.5 ± 1.9	32.1 ± 2.2	20.1 ± 0.06	14.3 ± 0.6	6.7 ± 0.9	41.0 ± 0.9
Glucuronic acid	1.1 ± 0.05	1.4 ± 0.1	1.9 ± 0.2	2.4 ± 0.1	1.3 ± 0.04	1.2 ± 0.1	–	–	–	–

[a] Data according to Junker et al. [18].
[b] Values represent yield in g/100 g raw material ± range/2 ($n = 2$).
[c] Values represent mean ± empirical standard deviation, $n = 3$.

Table 2
Relative composition (mol%) of arabino- and galacto-oligosaccharides enzymatically liberated from six differently extracted sugar beet pectins and insoluble (IDF) and soluble (SDF) dietary fibers of amaranth and quinoa seeds. Monosaccharides that were enzymatically released, too, but not diagnostic were not included in these calculations. Values represent mean ± empirical SD (n = 3). The structures of arabino- and galacto-oligosaccharides are given in Figs. 1 and 2. SD, standard deviation; n.q., detected but not quantifiable.

Oligo-saccharide	Pectin fraction						Amaranth		Quinoa	
	1	2	3	4	5	6	IDF	SDF	IDF	SDF
					Arabino-oligosaccharides					
A-2a	54.2 ±1.0	54.3 ±0.4	50.0 ±0.4	45.1 ±0.2	55.4 ±0.1	53.9 ±0.1	67.0 ±0.7	53.6 ±4.0	69.8 ±0.6	66.4 ±4.1
A-2b	–	–	–	–	–	–	2.4 ±0.1	6.9 ±1.5	–	–
A-3a	0.8 ±0.1	1.1 ±0.0	1.1 ±0.0	1.1 ±0.1	1.0 ±0.1	1.0 ±0.1	1.9 ±0.1	17.8 ±1.5	0.9 ±0.0	12.8 ±2.5
A-3b	2.8 ±0.5	4.2 ±0.1	10.8 ±0.5	46.1 ±0.1	3.0 ±0.0	4.8 ±0.1	1.0 ±0.0	–	1.2 ±0.0	–
A-4a	17.1 ±0.1	16.8 ±0.1	16.0 ±0.1	7.0 ±0.2	17.0 ±0.1	16.6 ±0.0	15.5 ±0.3	15.1 ±2.8	14.3 ±0.6	12.1 ±1.4
A-4b	1.5 ±0.0	1.3 ±0.1	1.4 ±0.1	0.7 ±0.1	1.5 ±0.0	1.4 ±0.0	1.8 ±0.2	n.q.	0.8 ±0.0	–
A-5a	1.5 ±0.0	1.7 ±0.0	1.7 ±0.1	–	1.7 ±0.0	1.6 ±0.0	1.2 ±0.0	2.6 ±0.2	0.8 ±0.1	1.4 ±0.1
A-5b	–	–	–	–	–	–	n.q.	–	1.4 ±0.1	4.4 ±0.1
A-5c	–	–	–	–	–	–	–	–	3.2 ±0.3	1.0 ±0.1
A-6a	5.4 ±0.2	4.8 ±0.1	4.4 ±0.1	–	4.8 ±0.0	5.0 ±0.0	2.4 ±0.2	n.q.	2.1 ±0.1	0.7 ±0.1
A-6b	3.6 ±0.6	3.8 ±0.2	6.4 ±0.2	–	3.0 ±0.0	3.9 ±0.1	–	–	–	–
A-7a	0.6 ±0.0	0.6 ±0.0	0.5 ±0.0	–	0.6 ±0.0	0.6 ±0.0	–	–	–	–
A-7b	12.5 ±0.2	11.5 ±0.0	7.7 ±0.3	–	12.1 ±0.1	11.2 ±0.0	6.7 ±0.1	1.9 ±0.2	5.7 ±0.2	1.3 ±0.3
					Galacto-oligosaccharides					
G-2a	96.7 ±0.2	96.5 ±0.5	97.1 ±0.2	97.9 ±0.1	96.6 ±0.1	96.8 ±0.1	100	100	–	–
G-2b	3.3 ±0.2	3.5 ±0.5	2.9 ±0.2	2.1 ±0.1	3.4 ±0.1	3.2 ±0.1	–	–	–	–
G-2c	–	–	–	–	–	–	–	–	–	–
G-3a	–	–	–	–	–	–	–	–	–	–
G-3b	–	–	–	–	–	–	–	–	–	–
G-4a	–	–	–	–	–	–	–	–	–	–
G-4b	–	–	–	–	–	–	–	–	–	–

arabinopyranose units (G-2b). Galactans in amaranth are solely linear whereas the galactan profiling of quinoa fiber did not release any previously described galactan structures.

A characteristic structural feature of arabinans and galactans from plants of the family Amaranthaceae is the esterification with *trans*-ferulic acid [13,15,16,24]. Thus, feruloylated oligosaccharides will also be released in these profiling approaches. Therefore, the approach to differentiate NFAOS and FAOS was applied to the described samples.

2.3. Application of the developed method to differentiate feruloylated and non-feruloylated arabino- and galacto-oligosaccharides

Based on the intensity of the ELSD-signal, both FAOS-fractions after *endo*-arabinanase cleavage and after *endo*-galactanase cleavage are minor if compared to the signals of the respective NFAOS-fractions. Although FAOS-fractions only make up a small portion of the totally liberated oligosaccharides, their contents allowed for the analysis by HPAEC-PAD after enzymatic deferuloylation. The resulting relative molar compositions of arabino- and galacto-oligosaccharides separated according to NFAOS and FAOS are summarized in Table 3. Regarding both the arabino- and galacto-oligosaccharides in NFAOS an almost identical composition compared to the oligosaccharides without preseparation according to feruloylation (Table 2) was observed. This result was expected as NFAOS make up the vast majority of liberated oligosaccharides in the profiling approaches. It is noteworthy that A-2b and A-3a contents in SDF of amaranth deviate if compared to the unseparated arabino-oligosaccharides (A-2b: 40.6 to 6.9 mol%, A-3a: 3.0–17.8 mol%). Deviations in A-3a proportions are comparably easy to explain as it is a marker for an incomplete enzymatic degradation that, in case of incomplete hydrolysis, shows up instead of A-2a; however, it represents the same structural unit. Differently, large amounts of A-2b are much harder to explain: A-2b is an α-1,5-linked-dimer consisting of an arabinofuranose and an arabinopyranose unit. Even if A-2b was originally isolated from amaranth ([28] HPAEC profiling) the portions detected here appear to be unusually high, an observation that we are currently unable to explain.

Analysis of the FAOS resulted in a different composition of the arabino-oligosaccharides (if compared to both oligosaccharides without preseparation and to NFAOS) as demonstrated by the oligosaccharide composition data across sample materials. In all samples, portions of higher oligomeric and more complex arabino-oligosaccharides tend to be larger compared to NFAOS. This can, for example, be seen by comparing the HPAEC-PAD chromatograms of arabino-oligosaccharides of FAOS and NFAOS liberated from Pct2 (Fig. 4): The chromatogram of the FAOS fraction shows larger signals after 70 min, which indicate the occurrence of higher oligomeric structures compared to NFAOS.

FAOS in sugar beet pectin show a similar acid lability as NFAOS: With increasing extraction time of the different pectins (under acidic conditions, Pct2-4), portions of A-4a, A-7a, and A-7b are reduced, indicating a debranching under acidic conditions. Simultaneously, contents of A-2a increase. Sonication has no distinct effect on feruloylated arabinan structures (compare Pct6 with Pct2). Also, feruloylated arabinans in the autoclaved Pct1 are built equally complex as shortly extracted Pct at pH 2.

Just as in sugar beets and as stated above, FAOS in amaranth and quinoa dietary fiber only make up a small portion of arabino-oligosaccharides, which was also indicated by signals with signal-to-noise ratios close to the limit of quantitation, especially if SDF was analyzed. Nonetheless, analysis of these samples also suggests that the liberated oligosaccharides in FAOS are more complex than those of NFAOS. It is also noticeable that FAOS contain high proportions of A-3a, A-3b and A-6b which were claimed to be side products of the enzymatic cleavage. Large contents of A-3a (16–31 mol%) in FAOS may indicate an influence of ferulates on enzymatic cleavage.

To study aforementioned effect in more detail, the FAOS arabino-oligosaccharides liberated from Pct2 were separately treated a second time with arabinanase in two different approaches, before and after deesterification. Thus, a potential impact of ferulate on the degradability of arabinans/arabino-oligosaccharides was aimed to study (Table 4). These data show that arabinanase is generally able to cleave feruloylated arabinans, however, probably somewhat slower. Such an effect was indicated by reducing portions of A-3a in both approaches compared to the control with a simultaneous increase of A-2a as end product. Portions of A-3a decrease from 26.0 mol% to 15.8 mol% when feruloylated arabino-oligosaccharides were treated with arabinanase and to 4.6 mol% in case that ferulates were removed prior to the arabinanase treatment. Thus, ferulates appear to have a hindering effect on arabinanase mediated arabinan degradation. It is noteworthy that A-7a and A-7b contents decrease as well, although these compounds are supposed to be inert to further arabinanase digestion. However, as only relative portions are considered it is also possible that higher oligomeric oligosaccharides (>7 arabinose units, not considered in the balance as not measurable but indicated by late signals in the chromatograms) are further degraded resulting in higher portions of smaller oligomeric compounds and, thus, decreasing the relative portions of A-7a and A-7b. Although this appears to be a valid assumption, it cannot reliably be verified because a decrease of these signals cannot visibly be detected and standard compounds that would represent these signals (>7 arabinose units) and allow for precise quantification are not available, neither commercially nor by isolation in the lab (yet). However, more generally, these data suggest that the incubation time for arabinanase digestion might be too short to degrade all feruloylated oligosaccharides to their arabinanase induced end-products. Doubling the incubation time to 48 h (Table 5 in combination with Table 3) leads to minor changes in the NFAOS composition, mainly small increases of A-3b and A-6b portions as a result of the prolonged impact of the arabinofuranosidase side activity are noticeable. Comparing the FAOS composition after 24 and 48 h of incubation shows a decrease of A-3a, A-4a, A-7a, and A-7b. Simultaneously, A-2a portion increases, indicating that the cleavage of feruloylated oligosaccharides is not finished after 24 h. However, higher oligomeric compounds (>7 arabinose units) are still visible after 48 h, either suggesting that even 48 h of fermentation do not suffice to reach the end point of enzymatic cleavage or, more likely, that ferulic acid is involved in highly complex structural units that cannot be cleaved by using arabinanase. But, methylation analysis of the FAOS and NFAOS fractions does not show more complex arabinan structural elements in FAOS (data not shown). Thus, our hypothesis that ferulates are incorporated in more complex structures cannot unambiguously be proven here. In any case, our studies show that a decent part of sugar beet arabinans may not be accessible for characterization following enzymatic cleavage without expanding the pool of standard compounds. However, isolation and characterization of these higher oligomeric standard compounds will certainly be most challenging.

Regarding galactan structures, a large difference between FAOS and NFAOS was observed (Table 3). Whereas NFAOS contain primarily the linear galactose dimer G-2a, feruloylated galacto-oligosaccharides of FAOS liberated from sugar beet pectins exhibit more complex profiles containing the additional compounds G-3a and G-4b. G-3a is a linear β-1,4-conjugated galacto-trisaccharide representing the same “structural unit” as G-2a (linear galactan), whereas G-4b has an additional galactopyranose unit attached at position *O*-6 of the terminal unit (see below). Sonication has no distinct effect on the structures of the extracted galactans (compare Pct2 and Pct6). However, extraction at pH 3 (Pct 5) results in simpler galactan structures indicating an extraction of more complex galactans from cell walls under acidic conditions (as also shown by the autoclave extracted pectin Pct1; extraction in water). Contrary, with increasing extraction times at pH 2, portions of G-4b increase up to 85.1 mol% in Pct4. This is supported by the results of methylation analysis: portions of 1,6-linked galactopyranose increases from 2.0% in Pct2 up to 9.0% in Pct4 (Table S1). As described for the FAOS fraction of arabinans, two enzymatic approaches were carried out to analyze whether feruloylation affects the cleavage of galactans by an

Table 3
Relative arabino- and galacto-oligosaccharide composition (mol%) in non-feruloylated (NFAOS) and feruloylated (FAOS) oligosaccharide fractions of six differently extracted sugar beet pectins (Pct) and insoluble (IDF) and soluble (SDF) dietary fibers of amaranth and quinoa seeds as a result of the treatment with endo-enzymes. Monosaccharides that were enzymatically released, too, but not diagnostic were not included in these calculations. Values represent mean ± empirical SD (n = 2). The structures of arabino- and galacto-oligosaccharides are given in Figs. 1 and 2. SD, standard deviation; n.q., detected but not quantifiable.

Oligo-saccharide	Pectin fraction (sugar beet)						Amaranth		Quinoa		Pectin fraction (sugar beet)						Amaranth		Quinoa	
	1	2	3	4	5	6	IDF	SDF	IDF	SDF	1	2	3	4	5	6	IDF	SDF	IDF	SDF
	Non-feruloylated oligosaccharides (NFAOS)										Feruloylated oligosaccharides (FAOS)									
	Arabino-oligosaccharides																			
A-2a	57.8 ±0.5	57.8 ±0.8	50.2 ±0.4	47.3 ±0.3	57.8 ±0.8	54.7 ±0.5	69.5 ±0.6	40.6 ±2.5	76.9 ±2.6	74.0 ±0.5	16.1 ±0.2	16.7 ±0.7	24.8 ±1.6	54.4 ±0.9	14.6 ±1.0	17.8 ±0.5	29.3 ±0.8	65.9 ±3.0	41.6 ±8.7	33.0 ±5.2
A-2b	–	–	–	–	–	–	2.3 ±0.1	41.2 ±3.0	–	–	–	–	–	–	–	–	–	–	–	–
A-3a	1.2 ±0.1	1.7 ±0.0	0.8 ±0.0	0.9 ±0.0	1.1 ±0.0	1.3 ±0.0	–	3.0 ±0.5	–	0.3 ±0.0	27.8 ±0.0	27.8 ±0.0	19.7 ±0.5	16.3 ±0.0	30.8 ±0.4	26.1 ±0.4	28.5 ±1.3	–	18.3 ±4.1	22.5 ±4.9
A-3b	1.8 ±0.3	3.7 ±0.2	14.5 ±0.2	44.5 ±0.2	2.7 ±0.0	6.4 ±0.3	–	0.7 ±0.1	–	1.4 ±0.4	5.1 ±0.0	6.7 ±0.1	9.5 ±0.1	14.2 ±0.7	5.3 ±0.1	6.8 ±0.2	4.5 ±0.2	–	3.1 ±0.0	34.5 ±3.9
A-4a	19.0 ±0.0	16.6 ±0.2	16.0 ±0.3	6.5 ±0.1	18.7 ±0.2	18.2 ±0.2	15.9 ±1.0	11.5 ±0.7	13.8 ±1.2	13.9 ±0.6	17.4 ±0.1	14.5 ±0.1	12.8 ±0.2	9.1 ±0.2	14.5 ±0.1	14.7 ±0.2	15.3 ±0.1	34.1 ±3.0	14.9 ±0.6	10.1 ±3.6
A-4b	–	1.4 ±0.0	0.6 ±0.0	0.8 ±0.2	–	–	2.2 ±0.1	–	1.0 ±0.3	–	–	–	0.8 ±0.0	1.7 ±0.0	–	–	–	–	–	–
A-5a	1.6 ±0.1	1.7 ±0.0	1.6 ±0.4	–	1.7 ±0.0	1.7 ±0.0	1.2 ±0.1	1.4 ±0.2	0.7 ±0.0	0.6 ±0.0	1.1 ±0.0	1.4 ±0.0	2.1 ±0.1	4.3 ±0.0	1.0 ±0.0	1.3 ±0.1	–	–	–	–
A-5b	0.2 ±0.0	–	0.4 ±0.1	–	0.1 ±0.0	–	–	–	0.9 ±0.0	3.5 ±0.1	–	–	–	–	–	–	–	–	–	–
A-6a	4.6 ±0.1	4.1 ±0.1	4.3 ±0.1	–	4.3 ±0.1	4.3 ±0.1	2.5 ±0.0	–	1.9 ±0.2	2.2 ±0.1	2.6 ±0.1	2.3 ±0.1	2.4 ±0.1	–	2.5 ±0.1	2.3 ±0.1	8.8 ±0.8	–	6.5 ±0.8	–
A-6b	1.9 ±0.1	2.6 ±0.1	4.3 ±0.1	–	1.9 ±0.0	2.8 ±0.0	–	n.q.	–	0.5 ±0.1	5.7 ±0.1	7.7 ±1.0	9.3 ±0.0	–	5.8 ±0.1	7.3 ±0.2	2.5 ±0.0	–	2.9 ±0.1	–
A-7a	0.5 ±0.0	0.5 ±0.0	0.4 ±0.0	–	0.5 ±0.0	0.4 ±0.0	–	–	–	–	3.4 ±0.1	3.9 ±0.2	2.7 ±0.2	–	4.2 ±0.0	3.6 ±0.3	0.3 ±0.1	–	n.q.	–
A-7b	11.5 ±0.3	10.0 ±0.3	7.1 ±0.1	–	11.3 ±0.5	10.3 ±0.5	6.5 ±0.3	1.4 ±0.2	4.7 ±1.0	3.7 ±0.5	20.8 ±0.2	19.1 ±0.4	15.9 ±0.3	–	21.4 ±0.3	20.1 ±0.4	10.9 ±1.3	–	12.6 ±3.1	–
											Galacto-oligosaccharides									
G-2a	97.1 ±0.2	97.0 ±0.1	97.1 ±0.0	97.7 ±0.1	96.7 ±0.2	96.5 ±0.4	100	100	–	–	64.9 ±5.6	29.0 ±7.4	25.5 ±0.1	8.3 ±0.2	51.9 ±4.5	29.7 ±2.6	100	100	–	–
G-2b	2.9 ±0.2	3.0 ±0.1	2.9 ±0.0	2.3 ±0.1	3.3 ±0.2	3.5 ±0.4	–	–	–	–	–	–	–	–	–	–	–	–	–	–
G-3a	–	–	–	–	–	–	–	–	–	–	22.9 ±2.9	23.7 ±0.3	15.1 ±0.4	6.6 ±0.6	24.5 ±0.5	20.5 ±0.1	–	–	–	–
G-4b	–	–	–	–	–	–	–	–	–	–	12.2 ±2.7	47.3 ±7.1	59.5 ±0.5	85.1 ±0.4	23.6 ±4.0	49.8 ±2.7	–	–	–	–

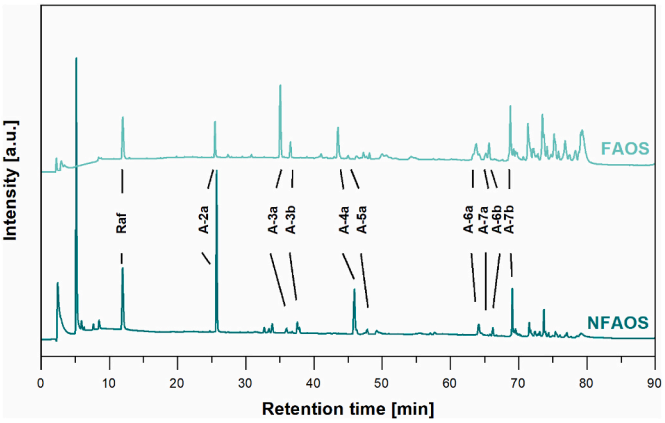

Fig. 4. Chromatograms of the feruloylated (FAOS) and non-feruloylated (NFAOS) oligosaccharide fractions of Pct2 liberated by arabinanase treatment and analyzed with high performance anion exchange chromatography coupled with pulsed amperometric detection. Raffinose (Raf) was used as internal standard. The structures of arabino-oligosaccharides are given in Fig. 1.

endo-galactanase (Table 4). The molar composition of the galacto-oligosaccharides did not change when the FAOS fraction was additionally treated with galactanase followed by feruloyl esterase. However, deesterification prior to a second galactanase treatment resulted in the loss of G-3a and an increasing portion of G-2a. This verifies that G-3a represents an intermediate product of galactanase treatment that cannot be cleaved by galactanase as long as ferulates are attached. Different from G-3a, G-4b was confirmed as a structural component of sugar beet galactans that cannot be cleaved by *endo*-galactanase, which to date has not been described.

Different from sugar beet, amaranth galactans exclusively released the linear feruloylated galactopyranose dimer G-2a (Table 3), which has been identified previously [13]. Moreover, feruloylated galacto-disaccharides were identified in spinach and sugar beet, too [29–31]. No feruloylated galacto-oligosaccharides were released from quinoa fibers.

2.4. Structural characterization of feruloylated galacto-oligosaccharides

To the best of our knowledge, feruloylated galacto-tri- and tetrasaccharides have not been detected to date; thus, data that characterize these compounds are not available. Because FAOS of Pct4 contain about 8% G-3a and 85% of the tetrasaccharide G-4b, the FAOS- of Pct4 were fractionated multiple times, and the fractions of interest were collected, concentrated and analyzed by RP-C18-MS^n. LC-MS data of feruloylated G-4b revealed a quasimolecular ion with *m/z* 865 ($[M+Na]^+$), which corresponds to the expected hexose based singly feruloylated tetrasaccharide. The ESI-MS^2 spectrum is shown in Fig. 5A. Losses of 60 and 120 Da result from ring cleavages of a 1,4-linked hexose [32,33]. Fragments with *m/z* 703 and 541 correspond to a ferulate moiety carrying a hexose trimer or dimer, respectively. To further clarify the location of the ferulate moiety, MS^3-analysis of the fragment ion with *m/z* 541 corresponding to a feruloylated hexose disaccharide was carried out (Fig. 5B). Losses of 60, 90 and, 120 Da confirmed the 1,6-linkage of the hexose. As no loss of ferulic acid or anhydro ferulic acid was observed it can be assumed that the ferulate is located on the terminal galactose. MS^4-analysis of the feruloylated terminal hexose revealed losses of 60, 90, and 120 Da, indicating the location of ferulate substitution at position *O*-6 of the terminal hexose as previously described for feruloylated galactans [15].

To further clarify the exact structure, two-dimensional NMR

Table 4
Relative portions of arabino-oligosaccharides (liberated from pectin 2 (Pct2)) and galacto-oligosaccharides (liberated from pectin 4 (Pct4)) in mol% in the feruloylated oligosaccharide fractions after three different enzymatic treatments. Values represent mean ± range/2 (n = 2). The structures of arabino- and galacto-oligosaccharides are given in Figs. 1 and 2. SD, standard deviation.

	Arabino-oligosaccharides									
	A-2a	A-3a	A-3b	A-4a	A-4b	A-5a	A-6a	A-6b	A-7a	A-7b
Arabinanase	20.3	26.0	7.0	14.6	0.6	1.3	2.7	7.0	3.7 ± 0.1	16.9 ± 0.1
→ Feruloyl esterase (control)	±0.2	±0.0	±0.0	±0.1	±0.1	±0.0	±0.1	±0.3		
Arabinanase	39.8	15.8	8.0	11.5	0.8	1.2	3.3	7.2	2.4	10.1
→ Arabinanase	±2.8	±0.2	±0.3	±0.5	±0.0	±0.1	±0.4	±0.8	±0.2	±0.8
→ Feruloyl esterase										
Arabinanase	48.8	4.6	5.3	20.2	0.8	1.5	2.9	4.7	1.4	10.0
→ Feruloyl esterase	±5.3	±0.7	±0.6	±2.0	±0.1	±0.2	±0.4	±1.3	±0.1	±1.7
→ Arabinanase										
	Galacto-oligosaccharides									
	G-2a	G-3a	G-4b							
Galactanase	5.0	7.7	87.4							
→ Feruloyl esterase (control)	±1.2	±0.4	±0.8							
Galactanase	5.2	7.8	87.0							
→ Galactanase	±1.1	±0.1	±1.0							
→ Feruloyl esterase										
Galactanase	10.9	–	89.1							
→ Feruloyl esterase	±3.2		±3.2							
→ Galactanase										

Table 5
Relative portions of feruloylated and non-feruloylated arabino-oligosaccharides from pectin 2 (Pct2) in mol% (n = 2) after incubation of Pct2 with *endo*-arabinanase for 48 h. Values represent mean ± range/2 (n = 2) The structures of arabino-oligosaccharides are given in Fig. 1 in the supplementary. SD, standard deviation.

	Arabino-oligosaccharides									
	A-2a	A-3a	A-3b	A-4a	A-4b	A-5a	A-6a	A-6b	A-7a	A-7b
FAOS	29.1	19.5	10.0	10.8	0.9	1.5	2.8	9.8	3.1	12.7
	±1.0	±0.2	±0.2	±0.0	±0.0	±0.1	±0.3	±0.2	±0.3	±0.2
NFAOS	53.4	0.5	7.7	17.3	0.7	1.2	4.5	4.7	0.5	9.6
	±0.5	±0.0	±0.0	±0.3	±0.1	±0.2	±0.1	±0.1	±0.0	±0.1

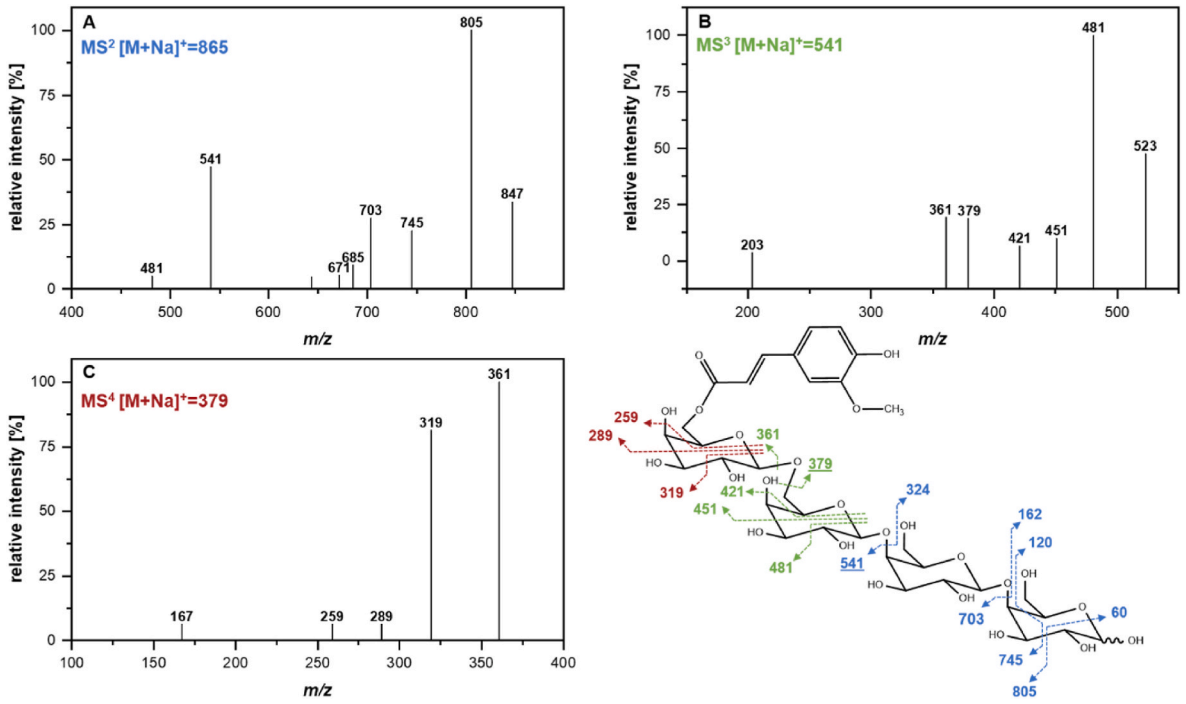

Fig. 5. ESI-MS^n mass spectra (positive mode) of the proposed feruloylated galacto-tetrasaccharide (*m/z* 865, sodium adduct).

experiments were carried out. The ^{1}H–^{13}C-HSQC spectrum is shown in Fig. S5. Due to low sample quantity, it was only possible to identify ferulate signals and signals of the anomeric protons, which were identified by comparing spectra with spectra of standard compounds of feruloylated G2a [13,29,30,34] and non-feruloylated G4b (Table S2), respectively. Due to low signal intensities interpretation of HMBC and ^{1}H–^{1}H–COSY spectra did not add useful information; for example, HMBC correlations to further confirm the position of the ferulate moiety were missing.

LC-MS^n-data of feruloylated G-3a ($[M+Na]^+ = 703$) followed a similar fragmentation pattern as described for feruloylated G-4b (Fig. S6), indicating that the ferulic substitution occurs at position *O*-6, too. However, since both the ions with $m/z = 541$ (MS^3) and $m/z = 379$ (MS^4) show mass losses of 60, 90, and 120 Da both are substituted at position *O*-6. This indicates the existence of two different, coeluting feruloylated G-3a isomers, both of which represent a linear β-1,4-linked galacto-trisaccharide: one that is feruloylated at position *O*-6 of the terminal hexose and one with the ferulic moiety being linked in position *O*-6 of the central hexose unit.

It is noteworthy that in addition to the identified feruloylated galactohexotri- and tetrasaccharides a feruloylated trisaccharide composed of two hexose and one pentose units ($[M+Na]^+ = 673$), presumably galactose and arabinose, was qualitatively identified via RP-C18-MS^3 in the Pct4 hydrolyzate (Fig. S7). LC-MS^2-data locates the pentose at the reducing end as indicated by losses of 60 and 90 Da, which is characteristic for a 1,4-linked pentose. Fragmentation of the ion with *m/z* 541 shows similar ions as described for feruloylated G-3a and G-4b. The existence of the ion with 379 Da confirms the location of a ferulic moiety at the terminal hexose. The losses of 60, 90, and 120 Da in the mass spectrum of the ion with *m/z* 541(MS^3) indicate a substitution of the second hexose at position *O*-6, too. However, the linkage between both hexoses is unknown, thus, besides ferulic acid, it is also possible that both hexoses are 1,6-linked, limiting the ferulate substitution to the terminal hexose.

3. Concluding remarks

This study presents a simple method to profile feruloylated arabino- and galacto-oligosaccharides in dicotyledonous plants after enzymatic cleavage. Interpretation of the data, however, requires consideration that feruloylation partially hinders full enzymatic degradation using *endo*-enzymes. Application of this method demonstrates, for example, that sugar beet, amaranth, and quinoa differ in terms of feruloylated galactans and their structures. Also, it reveals new feruloylated galactan structures in sugar beet that, however, do not exist in amaranth. Most likely this method can be expanded to other feruloylated oligosaccharides that need to be separated from their non-feruloylated counterparts, for example, feruloylated arabinoxylo-oligosaccharides.

4. Experimental

4.1. General

Dried sugar beet pulp (*B. vulgaris* L. var. *vulgaris*) that was kindly provided by Südzucker AG (Mannheim, Germany) was milled to pass a 0.5 mm sieve. Amaranth (*A. hypochondriacus*) and quinoa seeds (*C. quinoa* WILLD.) were obtained by a local supplier and were milled (<0.5 mm) and defatted with acetone before isolation of dietary fiber.

Unless otherwise stated all chemicals and reagents were of analytical purity grade and were purchased from VWR (Radnor, PA, United States), Sigma-Aldrich (Schnelldorf, Germany), or Carl Roth (Karlsruhe, Germany). Thermostable α-amylase (Termamyl 120 L, EC 3.2.1.1, from *B. licheniformis,* 120 KNU/g), the protease Alcalase (2.5 L, EC 3.4.21.62, from *B. licheniformis,* 2.5 AU/g), and an amyloglucosidase (AMG 200 L, EC 3.2.1.3, from *A. niger,* 300 AGU/g) were purchased from Novozymes (Bagsværd, Denmark). *Endo*-1,5-α-arabinanase (EC 3.2.1.99, *A. niger*) and *endo*-1,4-β-galactanase (EC 3.2.1.89, *A. niger*) and feruloyl esterases E-FAEZCT (EC 3.1.1.73, C. thermocellum, 7U/ml) and E-FAERU (rumen microorganism, 400 U/ml) were from Megazymes (Bray, Ireland). Ultrapure water was used throughout all experiments. Standard compounds of arabino- and galacto-oligosaccharides were previously isolated and characterized by our group [23].

4.2. Preparation of sample material

4.2.1. Extraction of feruloylated sugar beet pectins

Several Pct fractions were extracted as described in Junker et al. [18]. In brief: Pct 1 was obtained by an autoclave extraction using water (40 min, 121 °C, 2.1 bar). In addition to four differently acid extracted Pct, Pct 2 (pH 2, 1 h), Pct 3 (pH 2, 4 h), Pct 4 (pH 2, 16 h), and Pct 5 (pH 3, 4h), another Pct was extracted by applying additional sonication for 30 min prior to the acidification, followed by extraction for 1 h at pH 2 (Pct 6).

4.2.2. Isolation of amaranth and quinoa dietary fiber

Amaranth and quinoa seed flour (20 g) were suspended in 200 ml of sodium phosphate buffer (0.08 M, pH 6.0). Thermostable α-amylase (1.5 ml; Termamyl 120 L, EC 3.2.1.1, from *B. licheniformis,* 120 KNU/g) was added, and the suspension was heated for 30 min in a boiling water bath with occasional shaking. After cooling, the pH of the mixture was adjusted to 7.5 before adding 700 μl of Alcalase (2.5 L, EC 3.4.21.62, from *B. licheniformis,* 2.5 AU/g) and incubation for 30 min at 60 °C. The suspension was cooled down to room temperature and the pH was adjusted to 4.5. Amyloglucosidase (700 μl; AMG 200 L, EC 3.2.1.3, from *A. niger,* 300 AGU/g) was added, and the mixture was heated for 30 min at 60 °C with occasional shaking. The warm suspension was centrifuged for 15 min, and the residue was washed three times with water (60 °C). The supernatant and water wash solutions were combined and later used to isolate soluble dietary fiber fractions. The insoluble residue after centrifugation was washed twice each with ethanol and acetone before being dried overnight in an oven at 60 °C to yield insoluble dietary fiber (IDF). Soluble dietary fiber (SDF) was precipitated overnight after addition of ethanol to the combined supernatants described above (final ethanol concentration: 75 %). Precipitated SDF was recovered by centrifugation and washed with 80 % ethanol (v/v) and ethanol before being dried in an oven at 60 °C.

4.3. Characterization of sample materials

4.3.1. Monosaccharide analysis

Molar monosaccharide compositions of Pct and SDF fraction of amaranth and quinoa were determined according to de Ruiter et al. [35] with minor modifications: 6 mg of the sample was suspended in 0.5 ml of hydrogen-chloride in methanol solution (1.25 M) and methanolyzed for 16 h at 80 °C. A 10 μl aliquot was subsequently evaporated and hydrolyzed with 125 μl of a 2 M TFA solution at 121 °C for 1 h. The hydrolyzate was co-evaporated with ethanol, and the residue was dissolved in water and analyzed at 25 °C with HPAEC-PAD (ICS5000 system, Thermo Scientific Dionex, Sunnyvale, CA, USA) using a CarboPac PA200 column (150 × 3 mm i.d., 6.5 μm particle size, Thermo Scientific Dionex) and the chromatographic conditions given in Wefers et al. [16]. IDF fractions of amaranth and quinoa were analyzed according to Saeman et al. [36]: 10 mg of IDF was suspended with 150 μl of 12 M sulfuric acid in a glass vial and cooled down in an ice bath for 30 min with occasional shaking. Afterwards, the suspension was held at room temperature for 2 h with occasional shaking. After addition of 975 μl of water, the mixture was hydrolyzed for 3 h at 100 °C. The hydrolyzate was filtered through a 0.45 μm PTFE-membrane and after the addition of 75 μl of 4 M sodium hydroxide and 825 μl water analyzed with HPAEC-PAD as described above.

4.3.2. Methylation analysis

Polysaccharide linkage analysis of Pct fractions was carried out according to Gniechwitz et al. [37] and Wefers et al. [16]. In brief, 5 mg Pct or evaporated FAOS/NFAOS fraction was suspended in 2 ml of dimethyl sulfoxide overnight before 100 mg of freshly ground sodium hydroxide was added. After incubation for 90 min (sonication, room temperature), Pct were methylated using 1 ml of methyl iodide (30 min each, sonication, room temperature). Methylated Pct were extracted into 3 ml of dichloromethane, and the extract was washed with 5 ml of 0.1 M thiosulfate and 5 ml of water before being dried in a vacuum oven (40 °C). The methylation step was repeated to reduce undermethylation. Methylated Pct were hydrolyzed with 2 ml of 2 M TFA for 90 min at 121 °C. The solvent was evaporated, and the residual hydrolyzate was reduced by adding 0.3 ml of sodium borodeuteride in 2 M ammonia solution (20 mg/0.3 ml) for 1 h. To terminate the reduction, 100 μl of glacial acetic acid was added. Acetylation was carried out after adding 450 μl of 1-methylimidazol, 3 ml of acetic anhydride and incubation for 30 min at room temperature. Water (3 ml) was added, and partially methylated alditol acetates were extracted into 5 ml of dichloromethane. The organic phase was washed three times with 5 ml of water, and residual water was removed by freezing overnight at −18 °C. Analysis of partially methylated alditol acetates was carried out with GC-MS (GC-2010 Plus and GCMS-QP2010 SE, Shimadzu, Kyoto, Japan) equipped with a DB-225MS (30 m × 0.25 mm i.d., 0.25 μm film thickness, Agilent Technologies, Santa Clara, CA, USA) column and GC-FID (GC-2020 Plus) equipped with a DB-225 (30 m × 0.25 mm i.d., 0.25 μm film thickness, Agilent Technologies, Santa Clara, CA, USA) column. Chromatographic conditions are given in Wefers et al. [16]. For semi-quantitative analysis of partially methylated alditol acetates with GC-FID, molar response factors of Sweet et al. [38] were used.

4.4. Isolation, purification and characterisation of arabino- and galacto-oligosaccharide standard compounds

The in Figs. 1 and 2 depicted arabino- and galacto-oligosaccharides were isolated in earlier studies as described in Wefers and Bunzel [23]. The compound G-4b, which to date has not been described, was isolated after Driselase incubation of potato galactan as it was carried out for other galacto-oligosaccharides [23]. The compound was similarly characterized by MS- and NMR-experiments, especially with HSQC- and H,H-TOCSY experiments as described (Table S2).

4.5. Method development

4.5.1. Enzymatic liberation of oligosaccharides

Enzymatic liberation of arabino- and galacto-oligosaccharides was performed in duplicate as described by Wefers and Bunzel [23]: Pct fractions of sugar beet and SDF of amaranth and quinoa (100 mg) were suspended in 100 ml of water and incubated for 20 min at 95 °C in a water bath. After cooling down, 20 U of *endo*-1,5-α-arabinanase (EC 3.2.1.99, *A. niger*) or 100 U of *endo*-1,4-β-galactanase (EC 3.2.1.89, *A. niger*) were added. The mixture was incubated at 40 °C for 24 h (or 48h) under exclusion of light. In contrast, IDF of amaranth and quinoa were autoclaved first in order to increase the enzymatic accessibility: 100 mg of IDF was suspended in 10 ml of water and heated for 40 min at 121 °C in an oven. After cooling down to room temperature, 2 U of *endo*-1,5-α-arabinanase or 10 U of *endo*-1,4-β-galactanase were added and further treated as described above.

To inactivate enzymes, suspensions were heated for 5 min in a boiling water bath. Polymeric material was precipitated by addition of isopropanol (final isopropanol concentration: 66.7 %) and separated after centrifugation. The supernatant was evaporated to remove isopropanol, followed by a freeze-drying step.

4.5.2. Chromatographic separation of non-feruloylated and feruloylated oligosaccharides

To separate NFAOS and FAOS a semipreparative chromatographic method was established. Lyophilized arabino- or galacto-oligosaccharides were solubilized in water to obtain a defined concentration of 30 mg/ml. The solution was filtered through a PTFE membrane (0.45 μm) and a 100 μl-aliquot was separated by the application of high-performance liquid chromatography (HPLC) with UV detection (325 nm, Azura UVD2.1L, KNAUER, Berlin, Germany) using semi-preparative equipment (Azura P2.1L, KNAUER, Berlin, Germany). A

Luna phenylhexyl column (250 × 10 mm i.d., 5 μm particle size, Phenomenex Inc.) was used at a flow rate of 1 ml/min; the temperature was kept at room temperature. Additional detection was carried out by splitting the eluent 1/20 with the smaller fraction being directed to an evaporative light scattering detector (ELSD, Sedex 85, ERC GmbH, Riemerling, Germany) whereas the larger part was collected. Application of a programmable multiposition valve (Azura V2.1S, KNAUER, Berlin, Germany) allowed for fractionation. A binary gradient composed of water (A) and methanol (B) was used to separate NFAOS and FAOS: initially 80 % A and 20 % B held for 15 min, linear over 1 min to 20 % A and 80 % B held for 18.5 min, linear over 0.5 min to 80 % A and 20 % B, followed by equilibration for 15 min.

4.5.3. Identification of non-feruloylated and feruloylated oligosaccharides by LC-MS^n

Collected fractions of the released oligosaccharides after arabinanase or galactanase digestion were evaporated and redissolved in 200 μl of water before being analyzed with a Surveyor HPLC System coupled to a diode array detector and an LXQ linear ion trap MS^n system (Thermo Fisher Scientific, Waltham, Massachussetts, USA). Chromatographic conditions for NFAOS analysis were used as described by Wefers and Bunzel [23], applying an analytical PGC column (Hypercarb, 100 × 2.1 mm i.d., 3 μm particle size, Thermo Fisher Scientific) as stationary phase. FAOS were analyzed by using chromatographic conditions that were previously published by Schendel et al. [27], originally developed for the analysis of feruloylated arabinoxylan oligosaccharides on a Luna C18 column (250x4,6 mm i.d., 5 μm particle size, Phenomenex Inc.). Contrary to both mentioned methods, we omitted the reduction step of NFOS and FOS prior to HPLC analysis because LC-MS was solely carried out for qualitative purposes.

4.5.4. HPAEC-PAD/MS analysis

Prior to HPAEC-PAD analysis, NFAOS and FAOS fractions were evaporated as described above and dissolved in 200 μl of water. NFAOS were directly injected, whereas FAOS were digested with 2 U of feruloyl esterase from rumen microorganism (E-FAERU) and 0.035U of *Clostridium thermocellum* (E-FAEZCT) overnight at 40 °C in order to release ferulic residues. The enzymes were inactivated for 5 min at 95 °C, and the suspensions were centrifuged. Semiquantitative analysis of NFAOS and FAOS (following deesterification) was carried out according to Wefers and Bunzel [23] using an ICS-6000 system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a CarboPac PA200 column (250 × 3 mm i.d., particle size 5.5 μm, Thermo Fisher Scientific) using previously published response factors of arabino- and galacto-oligosaccharides. In addition, coupling of HPAEC-PAD with ESI-MS detection was used to confirm the assignment of standard compounds by scanning for their respective m/z ($[M+Li]^+$).

4.6. Influence of feruloylation on enzymatic liberation of oligosaccharides

Collected FAOS fractions were evaporated and dissolved in 200 μl of water before being treated in two different approaches with *endo*-arabinanase/*endo*-galactanase and feruloyl esterases, respectively. In the first approach, the collected fractions were digested a second time by adding 0.02 U of *endo*-arabinanase (Pct2) and 0.1 U of *endo*-galactanase (Pct4), respectively, and incubating for 24 h at 40 °C. After heating the solution at 95 °C for 5 min, the sample was cooled down to room temperature and 2 U of feruloyl esterase from rumen microorganism (E-FAERU) and 0.035 U of *Clostridium thermocellum* (E-FAEZCT) were added. The samples were then incubated overnight at 40 °C to deesterify the oligosaccharides. The inactivation of the enzymes was carried out by heating the solutions at 95 °C for 5 min. In the second approach the treatments with *endo*-arabinanase/*endo*-galactanase and feruloyl esterases were performed in the reverse order.

4.7. Isolation and characterization of feruloylated oligosaccharides

For the structural characterization of feruloylated galacto-oligosaccharides, multiple FAOS-fractions of Pct4 were collected, combined, evaporated and dissolved in a low amount of water to gain a highly concentrated solution, which was sufficient for MS- and NMR-characterization. LC-ESI-MS^n characterization was carried out after chromatographic separation using a Luna C18 column as described above (4.5.3).

NMR measurements were carried out on an Ascend 500 MHz NMR spectrometer (Bruker Biospin, Ettlingen, Germany) equipped with a Prodigy cryoprobe. The feruloylated oligosaccharide was dissolved in deuterium oxide and analyzed at 298 K by using 1D ^{1}H NMR and 2D H, H–COSY-, HSQC-, and HMBC-NMR-experiments using acetone for spectral calibration (δ_H: 2.22 ppm, δ_C: 30.89 ppm [39]).

CRediT authorship contribution statement

Florian Junker: Writing – original draft, Validation, Investigation, Conceptualization. **Mirko Bunzel:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Florian Junker reports financial support was provided by Graduate Funding from the German States (LGF). If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carres.2024.109076.

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