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A HILIC-PDA(-MS) profiling approach for the analysis of (methyl esterified) unsaturated galacturonic acid oligosaccharides released from pectins and food by-products

Rebekka Elke Schmidt, Mirko Bunzel *

Institute of Applied Biosciences, Department of Food Chemistry and Phytochemistry, Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany

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ABSTRACT

Functional and nutritional properties of pectins and pectic oligosaccharides (OS) depend on their structure. A major structural element of pectins are homogalacturonans, which consist of α-1,4-linked galacturonic acids (GalA). Homogalacturonans can be degraded to unsaturated (u)GalA-OS by pectin lyases. To analyze liberated uGalA-OS, a profiling approach based on hydrophilic interaction chromatography (HILIC) with photodiode array (PDA) and optional mass spectrometric (MS) detection was developed. Determination of molar relative response factors (RRF) of de-esterified uGalA-OS (degree of polymerization (DP) = 2–13) in relation to acarbose (internal standard) at 235 nm allows for (semi-)quantitative estimation of (methyl esterified) uGalA-OS and for application in other laboratories. Single quadrupole-MS enables to verify DP and to determine degree of methylation of uGalA-OS. Positions of methyl groups can be tentatively identified by orbitrap-MS. The developed profiling approach can be useful to analyze the specificity of a pectin lyase by analysis of uGalA-OS released from commercial pectin: The pectin lyase applied here appears to prefer a methyl group at cleavage subsite −1, but probably does not require a methyl group at subsite +1. In another application, various methyl esterified uGalA-OS (DP ≤ 12) were identified in enzymatically treated carrot pomace, a pectin-rich food by-product that can potentially be used to enhance nutritional properties of food products.

1. Introduction

Pectins are often the dominant polysaccharides of cell walls of dicotyledonous plants and mainly present in the middle lamella and the primary cell wall [1]. Structurally, pectins are a heterogeneous group of closely associated polysaccharides. Main structural elements are homogalacturonans and rhamnogalacturonans type I, the latter containing the neutral side chains arabinans and/or (arabino-)galactans type I [2]. Homogalacturonans consist of linear α-(1 → 4)-linked D-galacturonic acid (GalA) units, which can be methyl esterified at the carboxy group (C6) and/or acetylated at positions *O*2 and/or *O*3 [1]. On average, the chains of homogalacturonans from apples, citrus fruits, and sugar beets are thought to contain 72 to 100 GalA units. Both the degree of methylation (DM) and of acetylation (DAc) as well as the distribution of the methyl and acetyl groups across the molecule vary between plant species, maturation stage, and postharvest treatment, fundamentally determining the functional and nutritional properties of pectins. Regions of homogalacturonans that contain more than ten consecutive non-methyl esterified GalA units are able to form cross-links to other pectin molecules through calcium ions. These interactions may result in a compact package of pectins according to the so-called egg-box model, which is responsible for the formation of stable gels [1,3]. Therefore, pectins with a low DM tend to form gels at pH values of ≤6 in the presence of divalent ions (e.g., calcium ions). Differently, pectins with a high DM tend to form gels at pH values < 3.5 if high levels of soluble substances (e.g., sucrose) are present [4]. Thus, depending on the rheological properties of the pectins, they are mainly used as gelling agents, thickeners, and stabilizers in food industry.

In addition, various positive nutritional properties have been described for pectins and pectic oligosaccharides (OS) depending on their structures. Studies often focus on the prebiotic potential, which is rated higher for pectic OS than for pectins [5,6]. For example, GalA-OS improved both the growth of bifidobacteria and the production of short-chain fatty acids (SCFA) [7]. The SCFA pattern as well as specific changes in the gut microbiota of humans depend on the DM of GalA-OS and the presence of an Δ-4,5 unsaturated (u)GalA unit at the non-reducing end. GalA-OS promote the formation of propionate,

* Corresponding author.
E-mail address: mirko.bunzel@kit.edu (M. Bunzel).

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Glossary

DM	degree of methylation
DP	degree of polymerization
ESI	electrospray ionization
ELSD	evaporative light scattering detector
H2BC	heteronuclear 2-bond correlation (H2BC)
HPAEC-PAD	high performance anion exchange chromatography with pulsed amperometric detection
HILIC-PDA	hydrophilic interaction chromatography with photodiode array detection
HMBS	heteronuclear multiple-bond correlation
HSQC(-TOCSY)	heteronuclear single-quantum coherence (total correlation spectroscopy)
LMWSDF	low molecular weight soluble dietary fiber
LOD	limit of detection
LOQ	limit of quantification
MS	mass spectrometry
m/z	mass-to-charge ratio
RRF	relative response factors
SCFA	short-chain fatty acids
SIM	single ion monitoring
TFA	trifluoroacetic acid
TIC	total ion current
(u)GalA-OS	(unsaturated) galacturonic acids oligosaccharides

uGalA-OS the formation of butyrate. A high DM of (u)GalA-OS results in a slower fermentation [8]. Also, the DP of GalA-OS affects their prebiotic effect [9]. Moreover, GalA-OS (DP = 2–7) are expected to have an inhibitory effect on the adherence of bacteria to the epithelial cells and thus minimize the risk of infections [10].

There is increasing interest in the use of pectin-rich by-products of the agrifood industry to enrich food products with dietary fiber to sustainably improve their nutritional properties. For example, the production of juice and the extraction of sugar generate pectin-rich by-products such as apple and carrot pomace, citrus peel, and sugar beet pulp, respectively. To tailor the functional and nutritional properties of these by-products, they can be enzymatically treated [10]. For example, homogalacturonans can be hydrolyzed by polygalacturonanases or polymethylgalacturonanases resulting in GalA-OS, or degraded by β-elimination with pectin or pectate lyases resulting in uGalA-OS. The activity of these enzymes depends on the DM of the homogalacturonans [11].

Monosaccharides and OS are mainly analyzed by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). In principle, (u)GalA-OS can also be analyzed by HPAEC-PAD [12–15]. But, especially detection with PAD requires alkaline conditions, removing methyl and acetyl groups by saponification. Thus, (u)GalA-OS are often analyzed by capillary electrophoresis with various detectors such mass spectrometric (MS) detection [16,17]. Alternatively, hydrophilic interaction liquid chromatography (HILIC) on an amide-based stationary phase is a common approach, coupled to an evaporative light scattering detector (ELSD) or an MS detector [18,19]. However, due to the double bond in the structure, detection and especially quantification of uGalA-OS is also suitable using a photodiode array detector (PDA), usually at a wavelength of 235 nm [9,20]. Our aim was to develop a HILIC-PDA(-MS) based profiling approach to contribute to a better understanding of the fine structure of higher methylated homogalacturonans and present an alternative to the approach presented by Remoroza, Cord-Landwehr [19]. For this purpose, exclusively pectin lyase was chosen to enzymatically release uGalA-OS before analysis. Following alkaline hydrolysis, the developed method should be used to quantify uGalA-OS of varying DP released from homogalacturonans using molar relative response factors (RRF), and it was also deemed necessary to be able to analyze the number and position of methyl esters in the uGalA-OS. Our approach presented here enables the analysis of uGalA-OS after enzymatic degradation of isolated pectins or of plant cell wall materials.

2. Materials and methods

2.1. Materials

Pectin Classic CU201 (citrus pectin, 79 % GalA, DM = 69 %) was kindly provided by Herbstreith & Fox (Neuenbürg, Germany) and dried carrot pomace by Austria Juice (Kröllendorf, Austria). The carrot pomace consists of 60 % dietary fiber with the water-insoluble fraction (44 %) containing 18 % GalA (DM = 54 %) and the water-soluble fraction (16 %) containing 40 % GalA (DM = 85 %). Dietary fiber, GalA and DM were determined as described by Trabert, Schmid [21]. Commercially available, roughly purified pectin lyase (produced from a genetically modified organism), arabinanase, and cellulase, all of which are available for industrial applications, were used for enzymatic degradation. Sugar standards (arabinose, fucose, galactose, galacturonic acid, glucose, 2-desoxy-glucose, glucuronic acid, mannose, rhamnose, xylose) and all chemicals were of analytical grade and purchased from Alfa Aesar GmbH & Co KG (Karlsruhe, Germany), Carl Roth GmbH & Co. KG (Karlsruhe, Germany), Sigma Aldrich (Schnelldorf, Germany), or VWR Chemicals (Radnor, PA, USA).

2.2. Generation and isolation of unsaturated galacturonic acid oligosaccharides

2.2.1. Enzymatic depolymerization of pectin

Pectin Classic CU201 was dissolved in water (25 mg/mL) overnight at 30 °C while shaking (120 rpm). For enzymatic depolymerization of the pectin, pectin lyase (40 μL/g pectin) was added, and the solution was shaken for 4 h at 45 °C. Pectin lyase was inactivated by heating the solution to 90 °C for 5 min. A blank without addition of pectin lyase was treated similarly as a control. After partial removal of water by rotary evaporation, the fourfold volume of ethanol was added for precipitation of polysaccharides and large oligosaccharides. After 1–2 h, the suspension was centrifuged (10 min; 5000 rpm), and the supernatant was concentrated with rotary evaporation before freeze-drying.

To ensure the release and the preservation of uGalA-OS of various DP, both steps (enzymatic depolymerization, precipitation) were monitored for contents of uGalA-OS by HPAEC-PAD (Dionex ICS-5000 system, Thermo Fisher Scientific, Dreieich, Germany) using an analytical CarboPac PA200 column (250 × 3 mm, 6 μm particle size, Thermo Fisher Scientific) with pre-column (50 × 3 mm, 5.5 μm particle size, Thermo Fisher Scientific). The temperature was set to 25 °C, and the flow rate was 0.4 mL/min. An aliquot (25 μL) of each solution was injected. Ultrapure water (A), 100 mM NaOH (B), and 100 mM NaOH with 500 mM NaOAc (C) were used as eluents. After 10 min equilibration with 100 % C and 20 min with 90 % A and 10 % B, separation was performed by gradient elution: Starting with 90 % A and 10 % B, increasing B linearly to 50 % (0–10 min), further increase to 80 % B and 20 % C (10–20 min), followed by a linear increase to 100 % C (20–50 min), which was held until 75 min.

2.2.2. Alkaline hydrolysis of (methyl esterified) unsaturated galacturonic acid oligosaccharides

Due to different DP and DM values and variability in the distribution of methyl esters in enzymatically released uGalA-OS, there is a large number of different uGalA-OS in the hydrolysate. Hence, the product spectrum was reduced prior to fractionation by saponification of the methyl esters, with only DP being retained as a variable. Therefore, the mixture of freeze-dried uGalA-OS was solubilized in water (1:50 w/v) and cooled to 5 °C in the refrigerator. NaOH (1 M) was added to reach a

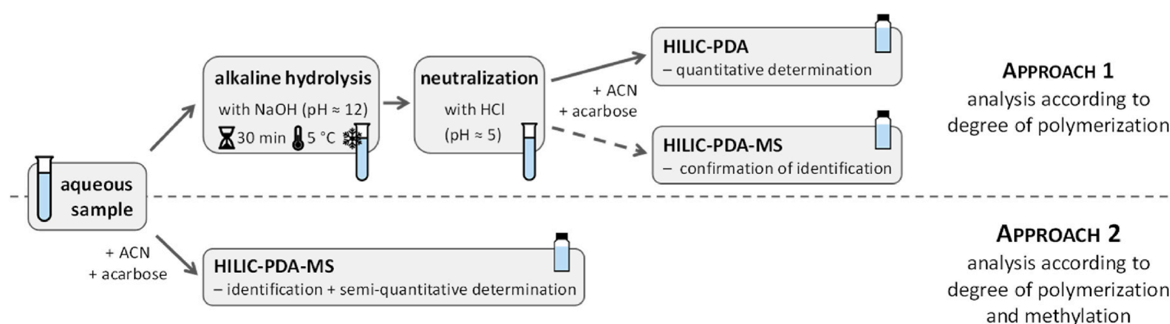

Fig. 1. Flow diagram of HILIC-PDA(-MS) based profiling approaches to identify and determine uGalA-OS after cleavage (approach 1) and with preservation (approach 2) of the methyl esters using acarbose as internal standard.

Fig. 2. *[1]H- (A) and heteronuclear single quantum coherence-NMR spectra (B) referenced against acetone ($\delta_H = 2.22$ ppm; $\delta_C = 30.89$ ppm) of the isolated unsaturated hexagalacturonic acid (structure in A). Highlighted in color are signals and structures of the unsaturated galacturonic acid unit (non-reducing end; pink), the galacturonic acid units in the middle (grey), and the reducing galacturonic acid unit (α-anomer: green/(β-anomer: blue).* (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

final concentration of 50 mM and a pH of about 12. After shaking, the solution was incubated for 30 min in the refrigerator (5 °C), shaking every 5 min. Immediately, the pH was adjusted to the initial pH of 5–6 by adding HCl (0.5 M). The supernatant of this de-esterified uGalA-OS

mixture was dried by rotary evaporation and freeze-drying. HILIC-PDA-MS, applying the method developed for de-esterified uGalA-OS (approach 1) described in section 2.4, was used to control the completeness of methyl ester saponification and to estimate the DP of the uGalA-OS by their mass-to-charge ratios (*m/z*).

2.2.3. Fractionation of unsaturated galacturonic acid oligosaccharides

The previously generated uGalA-OS were isolated using a preparative HILIC-UV (AZURA, Knauer) with an automatic fractionating valve with 16 ports. Separation was performed on an XBridge BEH Amide column (250 × 10 mm, 5 μm particle size, Waters Cooperation) with precolumn (10 × 10 mm, 5 μm particle size, Waters Cooperation). The temperature was set to 50 °C, the flow rate was 5 mL/min, and detection was performed at a wavelength of 235 nm. Aliquots (100 μL) of the diluted (1:2, v:v) freeze-dried alkaline hydrolyzed de-esterified uGalA-OS mixture were separated with a gradient of water (A) and ACN (B), both containing 0.2 % formic acid. In the first 30 min, B was reduced linearly from 70 % to 35 % and in the next 5 min to 30 %. After 2 min of isocratic elution, B was increased to 70 % over 1 min, and the column was equilibrated for 12 min. Twelve fractions (see Figure-S1) were collected according to the UV-detected chromatogram and dried by rotary evaporation and freeze-drying. The residue was dissolved in water and freeze-dried again to completely remove formic acid.

2.3. Characterization of isolated unsaturated galacturonic acid oligosaccharides

2.3.1. Monosaccharide composition

The monosaccharide composition was determined as previously described [22]. Briefly, 5 μL of a solution of each uGalA-OS (1 mg/mL) was evaporated, 250 μL of methanolic HCl (1.25 M) was added for methanolysis (16 h, 80 °C) and, after evaporation, 250 μL of trifluoroacetic acid (TFA, 2 M) was added for TFA-hydrolysis (1 h, 121 °C). The liberated monosaccharides were analyzed by HPAEC-PAD using 2-desoxy-glucose as internal standard under conditions described by Wefers, Gmeiner [22].

2.3.2. Analysis with NMR

Solutions of each isolated uGalA-OS (1 or 2 mg/mL) in deuterium dioxide with acetanilide (0.5 mg/mL) and acetone (0.5 or 1.0 μL) were prepared. NMR-spectra of each isolated uGalA-OS were acquired with a Bruker Ascend 500 MHz spectrometer (Bruker Corporation, Rheinstetten, Germany) equipped with a Prodigy cryoprobe. To calibrate the NMR-spectra, signals from the methyl group of acetanilide ($\delta_H = 2.17$ ppm, $\delta_C = 23.36$ ppm) were used in relation to acetone ($\delta_H = 2.22$ ppm, $\delta_C = 30.89$ ppm) following Gottlieb, Kotlyar [23]. ^{1}H-, heteronuclear single-quantum coherence (HSQC)-, HSQC-total correlation spectroscopy (TOCSY)-, heteronuclear multiple-bond correlation (HMBC)-, and heteronuclear 2-bond correlation (H2BC)-experiments were utilized for the structural analysis of the isolated uGalA-OS. All NMR-experiments were performed with standard Bruker pulse sequences: zg30 (^{1}H), hsqcedetgp (HSQC), hsqcetgpml (HSQC-TOCSY), hmbcgplpndqf (HMBC), and h2bcetgpl3 (H2BC). To reduce 2D-experiment times, non-uniform sampling (50 % sampling density) was applied.

Concentrations of the twelve isolated uGalA-OS were calculated relative to the signal of the methyl group of acetanilide in the ^{1}H NMR spectra. The relaxation delay was 35 s and the number of scans 64. Purity was calculated from the ratio of the concentration determined by NMR to the concentration based on the weight.

2.3.3. Analysis with HILIC-PDA(-MS)

Aqueous solutions of each uGalA-OS (1 mg/mL) were diluted with ACN and water to reach a final concentration of 100 μM in 50 % ACN. HILIC-PDA(-MS) analysis was performed using the method for de-esterified uGalA-OS (approach 1) described in section 2.4.

2.4. Method development

The developed profiling approach consists of two analytical approaches (see Fig. 1). In the first approach, the uGalA-OS are determined according to their DP after saponification of potential methyl esters. The required alkaline hydrolysis is performed with NaOH. Different temperatures (5–20 °C), hydrolysis times (0.5 and 2 h), and concentrations of NaOH (20–100 mM) were tested. A complete alkaline hydrolysis of methyl esters without detection of alkaline degradation products was achieved if the cooled sample (5 °C) was saponified using 50 mM NaOH (pH ~ 12) at 5 °C for 0.5 h, followed by immediate neutralization with 0.5 M HCl (final pH ~ 5–6). In the second approach, the amounts of the various methyl esterified uGalA-OS are determined semi-quantitatively after they have been separated according to their DP and number of methyl esters.

In both approaches, HILIC-PDA (Nexera X2 with SPD-M30A, Shimadzu) was performed on an Acquity UPLC BEH Amide column (150 × 2.1 mm, 1.7 μm particle size, Waters Corporation) connected to a precolumn (5 × 2.1 mm, 1.7 μm particle size, Waters Corporation). Samples were injected in 50 % ACN containing 1 mM acarbose as internal standard. The injection volume was 10 μL, and the flow rate was 0.4 mL/min. PDA detection was carried out in the wavelength range of 190–350 nm. Different temperatures (35, 40, 45, and 50 °C) were tested in order to obtain sharp, narrow peaks with the best result being achieved at 50 °C (see Figure-S2). Mixtures of ACN and water of 20:80 (A) and 80:20 (B) each containing 10 mM ammonium formate and 0.2 % formic acid were used as eluents.

To separate the twelve de-esterified uGalA-OS (DP = 2–13) (approach 1) the gradient program was as follows: 0–1 min isocratic elution with 90 % B, 1–34 min stepwise linear reduction of B over 76 % (1–5 min), 75 % (5–7 min), 60 % (7–12 min), and 35 % (12–32 min) to 20 % (32–34 min), 34–35 min isocratic elution with 20 % B, 35–36 min linear gradient back to initial conditions and equilibration until 43 min. The uGalA-OS are identified by comparison of the relative retention times (RRT) to acarbose as internal standard. To additionally verify the identity of uGalA-OS, HILIC-PDA is coupled to an electrospray ionization (ESI)-single quadrupole-MS (LCMS 2020, Shimadzu). Ionization is performed in the negative ion mode, and *m/z* ratios are measured in both total ion current (TIC, *m/z*: 500–1520) and single ion monitoring (SIM)-mode (*m/z* of each uGalA-OS (DP = 2–13), see Table-S1).

A second gradient program was used to analyze the methyl esterified uGalA-OS (approach 2). The elution starts with 100 % B, followed by linear reduction of B over 75 % (1–40 min) to 20 % (40–55 min), changing back to 100 % B in 1 min, and equilibration at initial conditions until 64 min. However, longer-chain methyl esterified uGalA-OS cannot be separated completely. Therefore, above mentioned MS detection is mandatory to allow for differentiation of all (methyl esterified) uGalA-OS based on their *m/z* (see Table-S2).

2.5. Method validation

All twelve isolated uGalA-OS (DP = 2–13) were used to validate the developed quantitative profiling approach 1 to determine de-esterified uGalA-OS following saponification. The purity of uGalA-OS was taken into account if the purity determined by NMR was below 90 % (uGalA-OS with DP = 2, 3, 11). All solutions (mixture of DP = 3–10, individual solutions of DP = 2, 11–13) used for validation were prepared independently three times in 50 % ACN and measured with HILIC-PDA. Coefficients of variation were calculated from these triplicate measurements in order to evaluate the precision of the method (see Table-S3). Molar RRF of the twelve isolated de-esterified uGalA-OS to acarbose were determined as previously described [24]. Limit of detection (LOD) and limit of quantification (LOQ) for each uGalA-OS were calculated from a signal-to-noise ratio of 3:1 or 10:1, respectively. Recoveries of the de-esterified uGalA-OS were determined using an aqueous solution with or without matrix, respectively. Low molecular weight soluble dietary

Table 1
Identity and purity of the isolated unsaturated galacturonic acid oligosaccharides (uGalA-OS) analyzed by HILIC-PDA at 235 nm and single quadrupole-MS and with NMR; *m/z* = mass-to-charge ratio, $_{2,\ 3,\ \ldots}$ = degree of polymerization of the uGalA-OS.

uGalA-OS	*m/z*	quasi-molecular ion	purity/%		contamination with other uGalA-OS
			HILIC-PDA (235 nm)	NMR	
$uGalA_2$	351.3	$[M-H]^-$	100	19	–
$uGalA_3$	527.4		100	80	–
$uGalA_4$	703.5		100	94	–
$uGalA_5$	879.6/ 439.3	$[M-H]^-/[M-2H]^{2-}$	98	90	$uGalA_4$
$uGalA_6$	1055.8/ 527.4		99	96	$uGalA_4$ + $uGalA_5$
$uGalA_7$	1231.9/ 615.4		96	94	$uGalA_4$ - $uGalA_6$
$uGalA_8$	1408.0/ 703.5		96	95	$uGalA_4$ - $uGalA_7$
$uGalA_9$	791.6	$[M-2H]^{2-}$	96	97	$uGalA_5$ - $uGalA_8$
$uGalA_{10}$	879.6		95	94	$uGalA_6$ - $uGalA_9$
$uGalA_{11}$	967.7		94	82	$uGalA_6$ - $uGalA_{10}$
$uGalA_{12}$	1055.8/ 703.5	$[M-2H]^{2-}/[M-3H]^{3-}$	95	92	$uGalA_6$ - $uGalA_{11}$
$uGalA_{13}$	1143.8/ 762.2		90	97	$uGalA_6$ - $uGalA_{12}$

fiber (LMWSDF)-fraction extracted from carrot pomace as described in section 2.6.2 was used as matrix, being a representative matrix for a possible application of the method (section 2.6.2).

2.6. Method application

2.6.1. Analysis of pectin lyase degradation products indicating lyase specificity

A solution of commercial citrus pectin was hydrolyzed with pectin lyase as described in section 2.2.1. The hydrolysate was diluted with ACN (final concentration: 50 %), acarbose was added (final concentration: 1 mM), and methyl esterified uGalA-OS were analyzed by the developed HILIC-PDA-MS profiling approach 2 for methyl esterified uGalA-OS (see section 2.4). The tetramer containing three methyl groups dominated the enzymatically released uGalA-OS (see section 3.3.1). To identify the position of the three methyl esters, this tetramer was fractionated with HILIC-PDA using pure eluents (water, ACN) without buffer salts. The collected eluates were combined, concentrated by evaporation, and analyzed by direct infusion in the Q Exactive™ Plus orbitrap-MS (Thermo Fisher Scientific) using ESI in negative mode and collision energy of −10 eV. To further study the position of the methyl groups in other released uGalA-OS, the whole hydrolysate was analyzed with orbitrap-MS (parameter see Table-S4) directly after chromatographic separation and detection with HILIC-PDA.

2.6.2. Analysis of unsaturated galacturonic acid oligosaccharides in enzymatically treated carrot pomace

Carrot pomace can be enzymatically treated to improve its technofunctional and nutritional properties when processed into food. Therefore, a suspension of 5 % carrot pomace was heated up to 45 °C for 1 h while shaking (120 rpm). Arabinanase, cellulase, and pectin lyase were added (20 μL of each enzyme/g carrot pomace) to degrade the major cell wall polysaccharides (arabinans, cellulose, homogalacturonans) in carrot pomace. Incubation was performed for 30 min in an incubator (45 °C) under shaking (120 rpm). For enzyme inactivation, the hydrolysate was heated in a water bath at 95 °C for 10 min. The

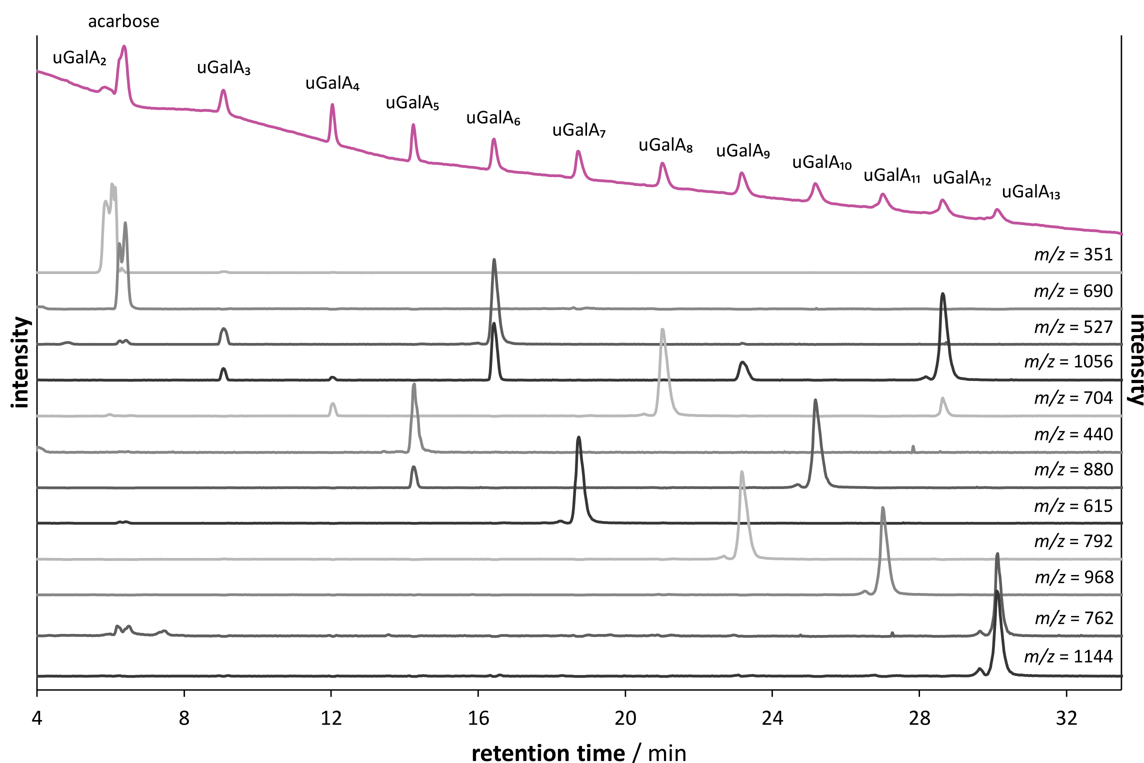

Fig. 3. Chromatogram sections at 235 nm (pink) and SIM-traces (grey) after separation of the twelve isolated de-esterified unsaturated galacturonic acid oligosaccharides (uGalA-OS) and acarbose (internal standard) with the developed HILIC-PDA-MS method (approach 1); *m/z* = mass-to-charge ratio of uGalA-OS ($[M-H]^-$, $[M-2H]^{2-}$ or $[M-3H]^{3-}$) and acarbose ($[M+\text{formate}]^-$), $_{2,\ 3\ \ldots}$ = degree of polymerization of the uGalA-OS. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

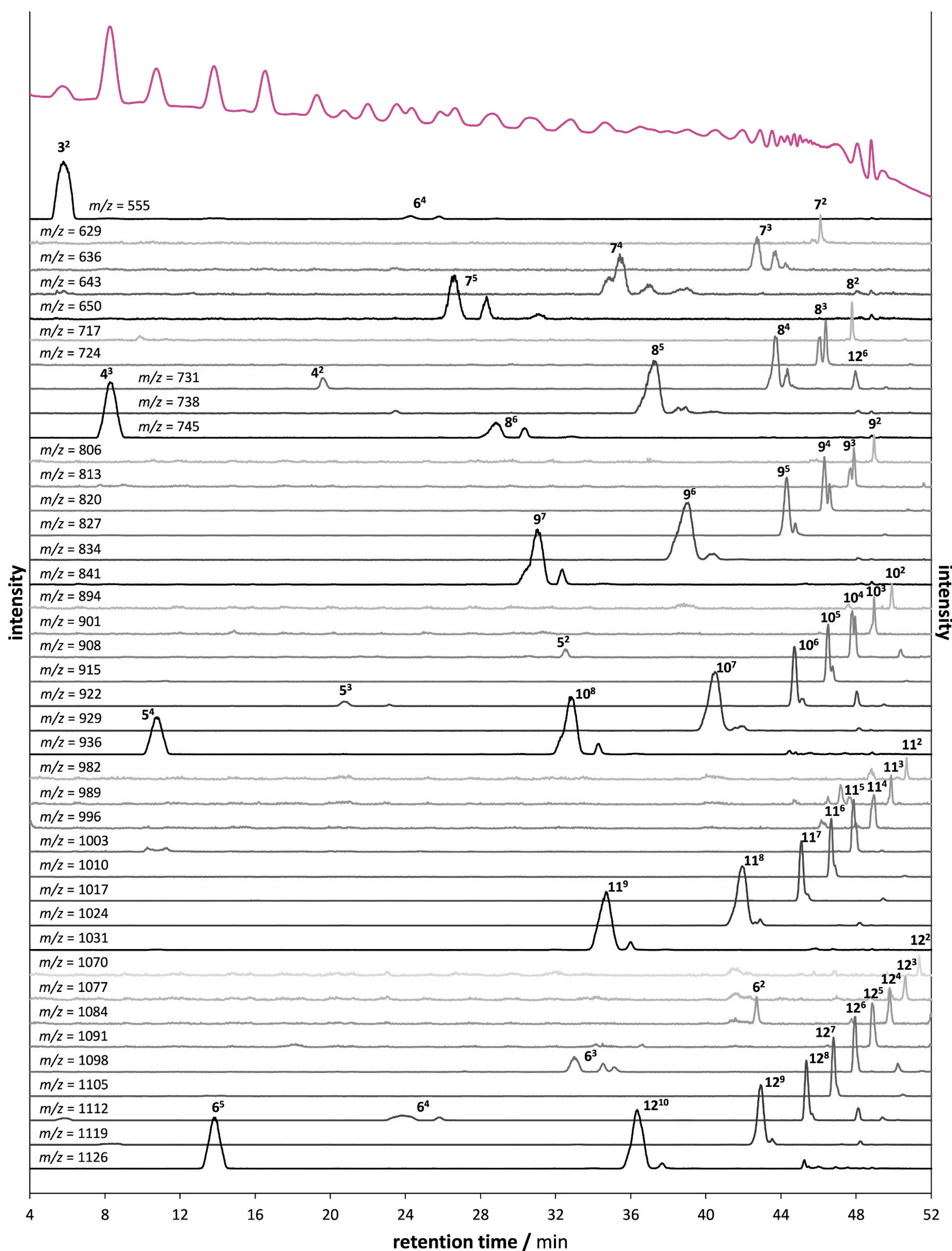

Fig. 4. Chromatogram sections of HILIC-PDA-MS approach 2 monitored at 235 nm (pink) and associated SIM-traces (grey) showing the separation of methyl esterified unsaturated galacturonic acid oligosaccharides (uGalA-OS) after enzymatic depolymerization of a commercial citrus pectin with the pectin lyase; 3, 4, …= degree of polymerization of uGalA-OS, $^{2,\ 3,\ \cdots}$= degree of methylation of uGalA-OS. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

hydrolysate was cooled, freeze-dried, and ground (≤500 µm). The LMWSDF-fraction was extracted using a buffer-free modification of AOAC-method 2017.16 [25]. That is, enzymatically treated carrot pomace and untreated carrot pomace (reference) were suspended in water (1 g/50 mL each), amyloglucosidase (200 µL/g carrot pomace) was added, and the suspensions were incubated for 30 min at 60 °C in a water bath while shaking. Insoluble dietary fiber was removed by centrifugation, and a fourfold volume of ethanol was added to the supernatant to precipitate high molecular weight soluble dietary fiber. After 30 min, the residue was removed by centrifugation, and the

Table 2
Validation data of the quantitative HILIC-PDA approach 1 for de-esterified unsaturated galacturonic acid oligosaccharides (uGalA-OS); $_{2,\ 3,\ \ldots}$= degree of polymerization of the uGalA-OS, RRT = relative retention time, RRF = molar relative response factor, LOD = limit of detection, LOQ = limit of quantification, recovery ± standard deviation (n = 3), matrix = low molecular weight soluble dietary fiber-fraction extracted from carrot pomace.

uGalA-OS	RRT	RRF	linear concentration range/µM	LOD/µM	LOQ/µM	recovery in water /%	recovery in matrix/%
$uGalA_2$	0.914	0.0723	28–82	7	9	86.2 ± 1.8	88.7 ± 2.2
$uGalA_3$	1.419	0.0368	8–80	2	4	107.0 ± 1.7	85.7 ± 6.0
$uGalA_4$	1.897	0.0382	10–100	2	3	102.9 ± 1.0	83.5 ± 6.6
$uGalA_5$	2.251	0.0403	10–100	2	3	100.8 ± 4.4	82.6 ± 6.0
$uGalA_6$	2.600	0.0379	10–100	2	3	103.9 ± 1.5	80.0 ± 3.8
$uGalA_7$	2.968	0.0374	28–100	2	3	104.4 ± 2.9	80.9 ± 7.6
$uGalA_8$	3.336	0.0401	28–100	2	4	104.5 ± 2.6	80.3 ± 7.5
$uGalA_9$	3.686	0.0419	10–100	2	4	102.8 ± 3.5	76.0 ± 5.3
$uGalA_{10}$	4.013	0.0478	10–100	2	4	100.0 ± 2.6	75.3 ± 5.6
$uGalA_{11}$	4.304	0.0485	23–82	3	5	92.7 ± 7.4	43.5 ± 41.2
$uGalA_{12}$	4.575	0.0529	28–100	4	6	92.6 ± 7.8	8.4 ± 52.1
$uGalA_{13}$	4.806	0.0632	28–100	4	9	77.5 ± 4.8	56.5 ± 82.8

Table 3
Partially methyl esterified unsaturated galacturonic acid oligosaccharides (uGalA-OS) released from a commercial citrus pectin by a commercial pectin lyase, the mass-to-charge ratios (*m/z*) of their quasi-molecular ions and dominant C- and Z-fragment ions according to the nomenclature suggested by Domon and Costello [31], and the loss of methanol (−32 (z = −1)/-16 (z = −2)); $_{3,\ 4,\ \ldots}$= degree of polymerization of uGalA-OS,$^{23\ \cdots}$ = degree of methylation of uGalA-OS.

uGalA-OS	quasi-molecular ion/*m/z* ([M − H]-/[M − 2H]$^{2-}$)	dominant C-/Z-fragment ions/*m/z* ([M − H]$^-$/[M − 2H]$^{2-}$)
$uGalA_3^2$	555 (−32)	175; 365
$uGalA_4^3$	745 (−32)	175; 365; 555
$uGalA_4^2$	731	175; 365; 541; 555
$uGalA_5^4$	935 (−32)	175; 365; 555; 745
$uGalA_5^3$	921	175; 365; 555; 731
$uGalA_6^5$	1125 (−32)	175; 365; 555; 745; 935
$uGalA_6^4$	1111 (−32)	175; 365; 541; 555; 731; 745; 921
$uGalA_6^3$	1097	175; 365; 541; 731; 907; 921
$uGalA_6^2$	1083	351; 731; 907
$uGalA_7^5$	650	175
$uGalA_7^4$	643	175; 365; 548
$uGalA_7^3$	636	175; 365; 541
$uGalA_8^5$	738	175; 365; 453; 548; 555; 643
$uGalA_8^4$	731	175; 365; 541; 548; 636
$uGalA_8^3$	724	175; 365; 534; 541; 629; 694
$uGalA_9^7$	840	175; 365; 460; 555; 650
$uGalA_9^6$	833	175; 365; 453; 548; 555; 643; 731; 738
$uGalA_9^5$	826	175; 365; 541; 555; 636; 731
$uGalA_9^4$	819	175; 365; 541; 629; 724
$uGalA_{10}^7$	928 (−16)	175; 365; 453; 548; 555; 643; 650; 738; 745; 833 (−16)
$uGalA_{10}^6$	921 (−16)	175; 365; 541; 555; 636; 731; 826
$uGalA_{10}^5$	914 (−16)	175; 365; 541; 555; 629; 636; 724; 731; 819
$uGalA_{11}^8$	1023 (−16)	175; 365; 453; 548; 555; 643; 738; 745; 833 (−16); 928 (−16)
$uGalA_{11}^7$	1016 (−16)	175; 365; 541; 555; 636; 731; 826 (−16); 921 (−16)
$uGalA_{11}^6$	1009 (−16)	175; 365; 541; 555; 629; 636; 724; 731; 819; 826; 914 (−16)
$uGalA_{12}^9$	1118 (−16)	175; 365; 5483 555; 643; 738; 745; 833; 928; 935; 1023 (−16)
$uGalA_{12}^8$	1111 (−16)	175; 365; 541; 555; 636; 731; 826; 921; 1016 (−16)
$uGalA_{12}^7$	1104 (−16)	175; 365; 541; 555; 731; 745; 819; 826; 914; 921; 1009 (−16)

supernatant (LMWSDF-fraction) containing LMWSDF (besides mono- and disaccharides) was dried by rotary evaporation and redissolved in water. The uGalA-OS of the extracted aqueous LMWSDF-fractions were analyzed with the developed HILIC-PDA(-MS) profiling approaches after saponification (approach 1) and with preservation of methyl esters (approach 2) (see section 2.4).

3. Results and discussion

3.1. *Characterization of isolated unsaturated galacturonic acid oligosaccharides*

To generate standard compounds, a commercial citrus pectin was treated with pectin lyase. Alkaline hydrolysis was performed in 50 mM NaOH in the cold to cleave methyl esters but to avoid additional degradation reactions. Following de-esterification, the uGalA-OS were isolated by preparative HPLC-UV using a HILIC mechanism (see section 2.2). Based on the *m/z* from HILIC-PDA-MS analysis, DP from 2 to 13 were assigned to the twelve isolated uGalA-OS (see Table 1). The monomer composition of the twelve uGalA-OS after acid hydrolysis showed, as expected, that the isolated uGalA-OS with a DP of 3, 6–10, 12, and 13 consisted exclusively of GalA (Figure-S3). The tetra- and pentamer fractions additionally contained lower molar proportions of glucose (<7 mol%) and the undecamer contained some additional glucose, xylose, and arabinose (<3–6 mol% each). The worst result was unexpectedly obtained for the isolated dimer, which consisted of only 20 mol% GalA. Thus, the data indicate impurities of these four isolated uGalA-OS with other monosaccharides and/or OS. These originate most likely from the pectic hydrolysate and were not completely separated during isolation.

Analysis by 1D and 2D-NMR allowed differentiation of the uGalA unit (non-reducing end, $\delta_H1 = 5.1$ ppm; $\delta_C1 = 100.3$ ppm, $\delta_H4 = 6.1$ ppm; $\delta_C4 = 112.2$ ppm), the GalA units in the middle ($\delta_H1 = 5.1$ ppm; $\delta_C1 = 100.6$ ppm), and the reducing GalA unit (α: $\delta_H1 = 5.4$ ppm; $\delta_C1 = 92.9$ ppm; β: $\delta_H1 = 4.7$ ppm; $\delta_C1 = 96.9$ ppm). On average, the individual uGalA-OS were mainly present as β-anomer (~57 %), with the α-anomer (~43 %, see Figure-S4) being minor. This ratio was calculated from the corresponding H1-signals of both anomers in the ^1H NMR spectra of the isolated uGalA-OS (DP = 4–13). Unsaturated di- and tri-GalA could not be considered since the H1-signal of the β-anomer was not isolated in the ^1H NMR spectra. Most of the other protons of the uGalA and GalA units could not be distinguished in the ^1H NMR spectra due to overlapping signals. Also, in the case of uGalA-OS with a DP ≥ 4, H1 and C1H1 signals (^1H or HSQC spectra, respectively) of the GalA units in the middle overlapped. Hence it was not possible to detect impurities of the twelve isolated uGalA-OS with uGalA-OS of another DP using NMR. In the dimer, the H4 of the uGalA unit showed a lower

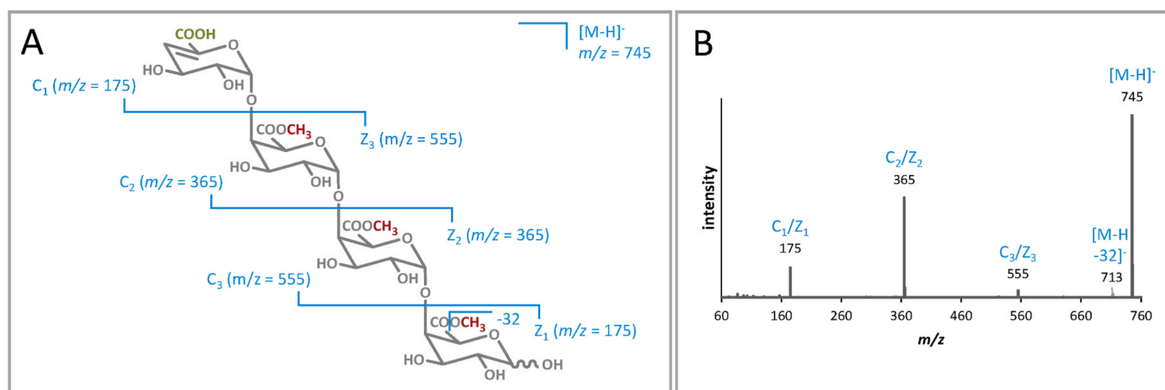

Fig. 5. Fragmentation of partially methyl esterified unsaturated tetragalacturonic acid by orbitrap-MS illustrated by (A) the structure and (B) the mass spectrum. Red: methyl groups, green: non-esterified carboxy group, $[M-H]^-$ = quasi-molecular ion, $C_{1\text{-}3}/Z_{1\text{-}3}$ = fragment ions according to the nomenclature suggested by Domon and Costello [31], −32: loss of methanol. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

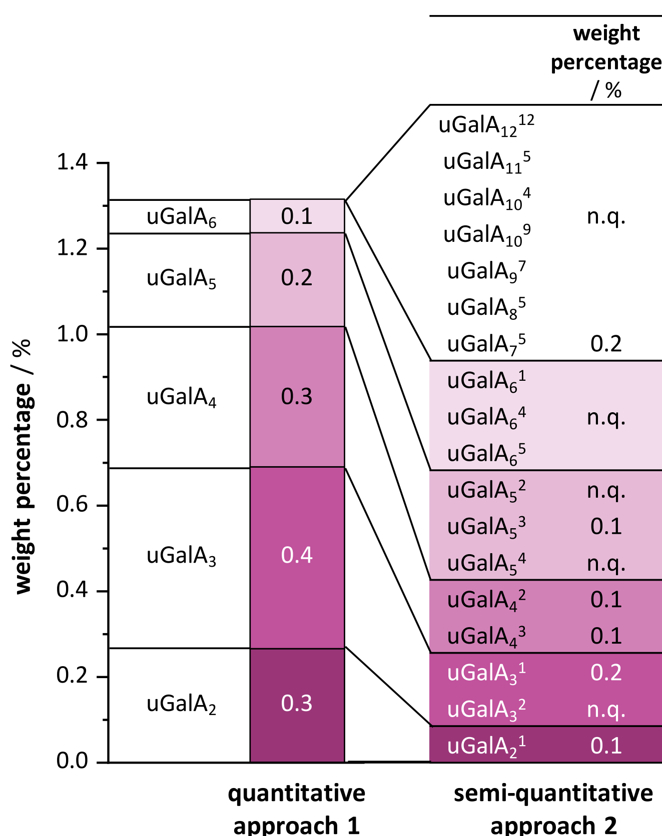

Fig. 6. Results of the analysis of the low molecular weight dietary fiber-fraction of enzymatically treated carrot pomace with the developed HILIC-PDA(-MS) profiling approach 1 for quantification following saponification and approach 2 for semi-quantitative estimation of de-esterified and methyl esterified unsaturated galacturonic acid oligosaccharides (u-GalA-OS), respectively; $_{2,\,3,\,\ldots}$= degree of polymerization of uGalA-OS, $^{1,\,2,\,\ldots}$= degree of methylation of uGalA-OS, n.q. = non-quantifiable.

chemical shift (δ_H1 = 5.8 ppm) compared to the other longer-chain uGalA-OS (see Figure-S4). Identified H1–H4/5 and C1H1–C4/5H4/5-signals in the ^{1}H- and HSQC-NMR-spectra are separated according to their assignment to the uGalA unit, the GalA units in the middle, and the reducing GalA unit, as exemplarily shown for unsaturated hexa-GalA in Fig. 2. The chemical shifts of the described signals are in accordance with the findings of Košťálová and Hromádková [26]. As an isolated characteristic signal in the ^{1}H-NMR-spectrum, the doublet of the H4 of the uGalA unit was selected for absolute quantification of the twelve isolated uGalA-OS (DP = 2–13) (see Fig. 2 and Figure-S4). For most of the uGalA-OS the purity determined by NMR was ≥90 %. The trimer and the undecamer had a lower purity of ≥80 % and the purity of the dimer was only 19 % (see Table 1). In summary, purities of the twelve uGalA-OS as determined by NMR are in accordance with the results of the monosaccharide composition described before.

When determining purity using PDA detection at 235 nm after chromatographic separation, only impurities with other substances absorbing at this wavelength are detected. Thus, saturated neutral and acidic monosaccharides and OS, for example, are not taken into account. However, different from the NMR analysis, the detection of impurities with shorter- and/or longer-chain uGalA-OS is possible. Contamination with other uGalA-OS increased with increasing DP. Nevertheless, the purity of all twelve isolated uGalA-OS determined by HILIC-PDA at 235 nm was ≥90 % and for most of them even ≥95 % (see Table 1).

3.2. Method development and validation

As shown in Fig. 1, two different approaches were used to characterize uGalA-OS: 1) separation of the de-esterified uGalA-OS (standard substances isolated and characterized as described in section 3.1) following saponification; 2) separation of the uGalA-OS still containing their native methyl esters. Whereas approach 1 gives reliant information about the DP and allows for a truly quantitative analysis, approach 2 results in additional structural information relating to existing methyl esters but can only be performed semi-quantitatively due to the lack of standard compounds for each methyl ester of the uGalA-OS (see below). For approach 1, saponification conditions are of upmost importance: complete alkaline hydrolysis of methyl esters without detection of alkaline degradation products by HILIC-PDA-MS was achieved if the pre-cooled samples were saponified for 30 min using 50 mM NaOH at 5 °C. While concentrations below 50 mM led to an incomplete saponification, higher concentrations resulted in a shift of uGalA-OS with a higher DP to those with a smaller DP due to β-elimination. Thus, it is important to 1) cool the sample to 5 °C before alkaline hydrolysis, 2) to immediately neutralize the alkaline hydrolysate, and 3) to monitor the pH during the process (pH ~ 12 after addition of NaOH, pH ~ 5–6 after neutralization). Higher temperatures must be avoided because longer-chain uGalA-OS are likely to be degraded to shorter-chain uGalA-OS by β-elimination. Accordingly, it was shown that evaporation in order to concentrate neutralized hydrolysates resulted in a loss of up to 90 % of individual uGalA-OS. We were therefore able to confirm previous data describing that β-elimination was favored against saponification with increasing temperature [27].

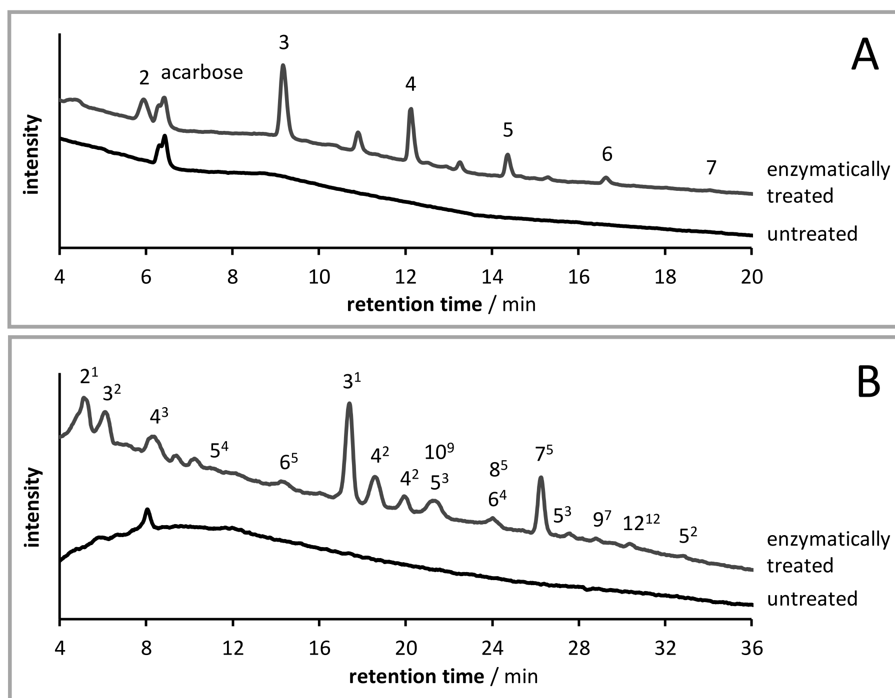

Fig. 7. Chromatogram sections of HILIC-PDA monitored at 235 nm of the low molecular weight dietary fiber-fractions of the untreated and enzymatically treated carrot pomace using the profiling (A) approach 1 for de-esterified unsaturated galacturonic acid oligosaccharides (uGalA-OS) following saponification and (B) approach 2 for methyl esterified uGalA-OS; acarbose = internal standard; 2, 3, …= degree of polymerization of uGalA-OS; $^{1,\ 2,\ \cdots}$= degree of methylation of uGalA-OS.

The chromatographic separation of uGalA-OS using a HILIC mechanism was developed based on literature data [18]. Different elution profiles were developed for both approaches as methyl esterification increases the number of analytes to be separated and largely affects the elution behavior. The more methyl esters a uGalA-OS of defined DP contains, the earlier it elutes (see Fig. 4), as also described by Leijdekkers, Sanders [18]. The ACN concentration of the injected sample solution did not affect the chromatographic separation (within the tested range of 30–70 % ACN), but a loss of longer-chain uGalA-OS due to precipitation was observed at 60 % ACN and more. Thus, the samples are prepared in 50 % ACN to enable the analysis of all uGalA-OS (DP = 2–13). For quantification purposes, acarbose was added as an internal standard. Acarbose does not occur naturally in plants, is UV-active at 235 nm, and can be separated chromatographically from the uGalA-OS.

Using the developed HILIC-PDA(-MS) method within approach 1, it is possible to separate twelve de-esterified uGalA-OS (DP = 2–13) within 30 min (see Fig. 3). These uGalA-OS can be identified by their RRT (see Table 2) and, if required, by their m/z of the $[M-H]^-$-, $[M-2H]^{2-}$- and/or $[M-3H]^{3-}$ quasi molecular ions in case of optional detection with MS. The dominating adduct-ion of acarbose is formed with formate ($[M + \text{formate}]^-$). In comparison to a previous HILIC-ELSD-MS based method [19] PDA and MS are connected in line and it is not necessary to split the eluate. For complex matrices, such as carrot pomace (section 3.3.2), additional detection with MS may be required due to coelution of uGalA-OS and UV-active matrix compounds and the comparatively low selectivity of the PDA at 235 nm. Quantification of the de-esterified uGalA-OS is performed by using molar RRF. Therefore, molar RRF for each uGalA-OS were determined as slope of the regression line after plotting the values of the product of the molar concentration (c) of the uGalA-OS and the area (A) of the acarbose signal against the product of the molar concentration of acarbose and the area of the uGalA-OS signal and setting the y-intercept to zero (see equation) (see Table 2).

$$molar\ RRF = \frac{c(uGalA-OS)*A(acarbose)}{c(acarbose)*A(uGalA-OS)}$$

These molar RRF can be used within the linear concentration range (approx.: 10–100 μM) to analyze the amount of uGalA-OS without the need of standards. This approach offers a good alternative to the previously introduced mathematical equation to quantify (u)GalA-OS only based on the ELSD responses of mono-, di- and tri-GalA-OS [19]. Validation data of the quantitative approach 1 are shown in Table 2. Coefficients of variation (see Table-S3) were across the calibrated linear concentration range mostly low (≤5 %), indicating a high precision of the measuring. However, coefficients of variation increase for some uGalA-OS at lower (≤15 %) and higher molar concentrations (≤7 %), thus showing decreased precision. LOD (2–4 μM, 7 μM ($uGalA_2$)) and LOQ (3–6 μM, 9 μM ($uGalA_2$ and $uGalA_{13}$)) are comparable for all analyzed uGalA-OS. LOD and LOQ tend to be higher for $uGalA_2$ and longer-chain uGalA-OS. For the latter, this can potentially be explained by a reduced solubility of longer-chain uGalA-OS in water and ACN. It is assumed that longer-chain uGalA-OS may partially precipitate (and potentially re-solubilize) before and/or on the column, resulting in broader signals in the chromatogram and thus lower signal-to-noise ratios. Recoveries of ten uGalA-OS (DP = 3–12) from an aqueous solution were between 90 % and 107 %, mostly in the range of 95–105 %. Only recoveries of $uGalA_2$ (86 %) and $uGalA_{13}$ (78 %) were lower. The lower recoveries of longer-chain uGalA-OS were probably also caused by their reduced solubility. Recoveries of uGalA-OS from matrix (here simulated by using LMWSDF-fraction from carrot pomace) show a comparable pattern as those from water although being generally lower (≤83 %). Hence, interactions between uGalA-OS and matrix compounds are assumed. These interactions might increase with the chain length of the uGalA-OS as indicated by the (very) low recoveries of uGalA-OS with a DP > 10. This, however, does not explain the surprisingly low recovery of $uGalA_{12}$ (8.4 %) from matrix in comparison to those of $uGalA_{11}$ and $uGalA_{13}$ (44 and 57 %, respectively). The high coefficients of variation (41–83 %) of the recoveries of these longer-chain uGalA-OS (DP = 11–13) indicate a low precision and suggest a low reproducibility of the observed matrix interactions. In general, however, the validation data for the developed HILIC-PDA profiling approach of de-esterified

uGalA-OS are deemed acceptable for uGalA-OS with a DP of 3–10. Differently, however, determination of longer-chain uGalA-OS (DP = 11–13) was associated with greater uncertainties possibly due to the low recovery associated with low precision in matrix ($uGalA_{11}$ - $uGalA_{13}$). In case of $uGalA_2$ the purity of the standard was too low for a successful validation.

In approach 2, the developed chromatographic method in combination with a mass spectrometric detection (see Table 3) enables the identification of most signals obtained from the analysis of enzymatically treated commercial citrus pectin, as shown in Fig. 4. A quantitative analysis, however, is difficult. The UV response (235 nm) of uGalA-OS with and without methyl esters of the same DP was found to be comparable for unsaturated tetra-GalA. In addition, the UV response (235 nm) was independent of the amount of ACN in the gradient program as shown for unsaturated tetra-GalA. Thus, the molar RRF determined for the de-esterified uGalA-OS can be used for a semi-quantitative estimation of the corresponding methyl esterified uGalA-OS. However, although peaks can nicely be identified by MS, the peak separation in combination with peak shapes and peak heights appears not acceptable for a truly quantitative approach, especially at a higher DP.

3.3. Method application

3.3.1. Analysis of pectin lyase degradation products indicating lyase specificity

Enzymatic depolymerization of a commercial citrus pectin with pectin lyase released uGalA-OS of DP = 3–12 with varying DM, as shown in Fig. 4. Intensities of the signals at 235 nm suggest that $uGalA_4^3$ was the dominant uGalA-OS released, followed by $uGalA_5^4$ and $uGalA_6^5$. Release of uGalA-OS decreased with increasing chain-length and decreasing DM. Independent of the DP, uGalA-OS with less than two methyl groups were not detected with the developed HILIC-PDA-MS approach. Hence, two methyl esterified carboxy groups within the homogalacturonan regions appear to be mandatory for cleavage by the pectin lyase. It is also assumed that the cleavage of homogalacturonans by the pectin lyase was more effective in highly methylated regions, since the majority of released uGalA-OS contains only one or two non-esterified carboxy groups. Fully methylated uGalA-OS were not detected, possibly because they may not ionize well in the negative ESI mode [28].

A chromatographic separation of uGalA-OS with the same DP and DM, but a different location of methyl groups has not been possible yet if only chromatographic separation and simple MS was applied. However, Leijdekkers, Huang [29] were able to differentiate between isomeric uGalA-OS with differently distributed methyl groups by traveling-wave ion mobility MS. Quéméner et al. (2003) characterized isomeric, partially methyl esterified GalA-OS after isolation with HPAEC and labeling of the reducing end with ^{18}O by iontrap-MS (MS^n), and methods using a direct analysis of mixtures of partially methyl esterified uGalA-OS using quadrupole-iontrap-MS were described as being suitable, too [28,30]. Here we demonstrate that analysis with orbitrap-MS may also be useful for the identification of the position of methyl groups in uGalA-OS. For this purpose, the main enzymatic degradation product, $uGalA_4^3$, was isolated and analyzed using orbitrap-MS to identify the cleavage position of the pectin lyase. Fragmentation in the ESI negative mode mainly results in ions of the C- and Z-series according to the nomenclature suggested by Domon and Costello [31] as previously described [28,30,32]. After the fragmentation of $uGalA_4^3$, three characteristic fragments (m/z = 175, 365, 555) were detected in addition to the quasi-molecular ion (m/z = 745), as shown in Fig. 5. The fragments were identified either as C_1- to C_3- or Z_1-to Z_3-fragments according to the suggested nomenclature [31]. Higher collision energies (−15, −20 and −25 eV) led to greater fragmentation and thus to more complex mass spectra and a higher proportion of small fragments. The m/z-ratios of the three fragment ions suggested that the non-esterified carboxy group was located either at the unsaturated non-reducing end or at the reducing end of the unsaturated tetra-GalA. Additionally, a loss of one molecule methanol (-32 Da) was found. According to the findings of Quéméner et al. (2003) the methyl group is preferentially released from the reducing end [30]; however, different from our study they analyzed partially methyl-esterified oligogalacturonides after depolymerization using an *endo*-polygalacturonase. Transferring the results of Quéméner et al. (2003) [30] to our study suggests that the non-esterified carboxy group was located at the non-reducing end. Consequently, the pectin lyase used appears to accept a non-esterified carboxy group at cleavage subsite +1 and prefers a methyl group at cleavage subsite −1. In contrast, previous investigations with another pectin lyase, isolated and purified from *Aspergillus niger* (strain 4 M − 147), showed that the carboxy group of the uGalA units (non-reducing end) of uGalA-OS released by the pectin lyase should be methyl esterified [33]. Other investigations with a pectin lyase from *Aspergillus niger* showed that the carboxy group at the non-reducing end can be either methyl esterified or not [28].

Additional released methyl esterified uGalA-OS were characterized by HILIC-PDA-orbitrap-MS after chromatographic separation of the hydrolysate derived from a commercial citrus pectin (see section 2.6.1). The dominating m/z and the mass spectra obtained are shown in Table 3 and Figure-S5, respectively. Depending on the DM of the uGalA-OS, the number of possible positions of the methyl esters increases with the DP, resulting in more complex mass spectra. This is due to the fact that uGalA-OS with the same DP and DM cannot be separated chromatographically, resulting in mass spectra that represent more than one compound. Additionally, quasi-molecular ions of uGalA-OS and their fragments with a DP of ≥5 resp. 6 mostly carried a double negative charge. Therefore, C_3- and C_6-fragments, for example, have the same m/z, complicating an unambiguous identification of the positions of the methyl esters in these uGalA-OS. Additionally, a differentiation of the isobaric C- and Z-fragments was not possible. However, again, a loss of methanol (-32 (z = −1)/-16 (z = −2)) suggests the location of a methyl group at the reducing end.

3.3.2. Analysis of unsaturated galacturonic acid oligosaccharides in enzymatically treated carrot pomace

Whereas uGalA-OS were not detected in the LMWSDF-fraction of the untreated carrot pomace, the LMWSDF-fraction of the enzymatically treated carrot pomace contained 1.3 % uGalA-OS (see Fig. 6). Mainly, unsaturated dimeric, trimeric, and tetrameric GalA-OS, but also smaller amounts or traces of longer-chain uGalA-OS (DP = 5–7) were present. These quantitative data are based on the profiling approach 1, that is, analysis of the uGalA-OS following de-esterification. The data can supplement the determination of LMWSDF (OS with DP ≥ 3) by AOAC-method 2017.16, since acidic OS such as uGalA-OS are not captured as LMWSDF by the AOAC-method [34]. Additional analysis with the HILIC-PDA-MS profiling approach 2, that is, analysis of the methyl esterified uGalA-OS, allowed the identification of uGalA-OS up to a DP of 12 with various methyl esters (see Fig. 7). Using this approach 2, it was also possible to determine some methyl esterified uGalA-OS semi-quantitatively (see Fig. 6). The overall content of methyl esterified uGalA-OS was semi-quantitatively estimated to 0.8 %. This amount is lower as the amount of uGalA-OS in the quantitative approach 1, among others, because several uGalA-OS were only detected in non-quantifiable traces by approach 2. Again, fully methylated uGalA-OS may not be ionized in the ESI negative mode, potentially contributing to this data, too. However, this application demonstrates nicely the suitability of the developed HILIC-PDA(-MS) profiling approach to analyze uGalA-OS: it is possible to quantitatively determine the de-esterified uGalA-OS (approach 1), thus getting information about the total amount of uGalA-OS and their distribution according to DP in a sample. In addition, analysis of the methyl esterified uGalA-OS (approach 2) gives us deeper insights into the structural characteristics (existing methyl esters) of the uGalA-OS, and in some cases, allows for a rough estimate of the specific species. For this purpose the use of MS^1 was sufficient, which makes the presented approach compared to that of Remoroza, Cord-Landwehr [19] less complex. Thus, the

developed approach is a useful tool to better understand the nutritional and functional properties of uGalA-OS and preparations containing uGalA-OS since both the nutritional and functional properties of uGalA-OS depend on their DP and DM [4,8,9].

4. Conclusion

The developed HILIC-PDA(-MS) based profiling approach allows for a quantitative analysis of uGalA-OS categorized according to their degree of polymerization. This analysis requires de-esterification of potential methyl esters. The de-esterification process developed here minimizes undesired degradation under the required alkaline conditions such as β-elimination. The advantage of this approach is therefore a precise quantification of the de-esterified uGalA-OS. However, as the methyl esterification potentially affects techno-functional and physiological effects of the uGalA-OS additional structural information can be achieved by using the analysis of the methyl esterified uGalA-OS, which requires MS detection. Interpretation of the mass spectra allows to acquire structural details of the uGalA-OS such as DP and DM. However, using this approach, a strict quantification is not possible, but a semi-quantitative estimation. Thus, the combination of both approaches is ideal to analyze unknown (fiber) samples.

CRediT authorship contribution statement

Rebekka Elke Schmidt: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Data curation. **Mirko Bunzel:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Rebekka Schmidt reports financial support was provided by German Ministry of Economic Affairs and Climate Action (BMWK). 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carres.2026.109816.

Data availability

Data will be made available on request.

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