Carbohydrate Research 344 (2009) 1808–1817

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Fingerprinting complex pectins by chromatographic separation combined with ELISA detection

René Verhoef [a], Yu Lu [a], J. Paul Knox [b], Alphons G. J. Voragen [a], Henk A. Schols [a,*]

[a] Laboratory of Food Chemistry, Agrotechnology and Food Sciences Group, Wageningen University, Bomenweg 2, 6703 HD Wageningen, The Netherlands
[b] Centre for Plant Sciences, University of Leeds, Leeds LS2 9JT, United Kingdom

ARTICLE INFO

Article history:
Received 25 June 2008
Received in revised form 13 September 2008
Accepted 24 September 2008
Available online 7 November 2008

Keywords:
MHR
Rhamnogalacturonan I
ELISA
Monoclonal antibody
HPSEC
WAX

ABSTRACT

Enzyme-resistant pectin or modified hairy regions were subjected to size exclusion (HPSEC) and weak anion exchange (WAX) chromatography. Fractions collected after separation were tested for the presence of different pectic epitopes using the monoclonal antibodies LM2, LM5, LM6, and JIM7. Separation by HPSEC showed that based on molecular weight the different epitopes were restricted to distinct molecular weight populations. WAX chromatography resulted in an even better separation of the different pectic epitopes present. A clear separation between arabinogalactan type II epitopes and the RG I side chains, (1,5)-α-L-arabinan and (1,4)-β-D-galactan, could be established. Arabinogalactan type II was found in the first populations eluting off the WAX column. The observations made within the ELISA assays of the collected fractions could be confirmed by determination of the sugar composition of the individual populations obtained. The sugar composition of the AGII positive populations eluting off the WAX column shows the presence of significant amounts of rhamnose and galacturonic acid. Together with the delay on an anion exchanger, this observation indicates a possible linkage between RGI and AGII. The volume of the individual fractions collected provides enough material for a maximum of 20 different antibodies to be tested from one analytical separation.

1. Introduction

Nowadays pectin is more and more recognized for its potential bioactive properties. Studies by Kokkonen et al., Morra et al., and Bussy et al.[1–3] have shown that surface coated modified hairy regions (MHRs) from different sources show adhesion stimulating effects toward mammalian bone cells. Within these studies, cell adhesion is suggested to be affected by the length of the side chains. However, the nature of the different side chains known to be attached to rhamnogalacturonan I, the main constituent of MHR, has not been taken into account.

MHR is obtained by liquefaction of different fruits and vegetables using enzyme mixtures rich in polygalacturonase, hemicellulases, and cellulases.[4] As a result of this liquefaction process, an enzyme-resistant pectic sample is obtained. This fraction mostly contains RGI, some residual homogalacturonan (HG), xylogalacturonan (XGA), and RGII. Both homogalacturonan and xylogalacturonan are built of a (1→4)-α-D-GalpA backbone. Xylogalacturonan is HG, substituted by single β-D-Xyl*p*-(1→3) units.[5] Both HG and XGA can be methyl esterified at position C-6, HG can be substituted by *O*-acetyl moieties at the O-2 and/or O-3 position.[6–8]

Rhamnogalacturonan I has a backbone of →2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→ repeats branched at position O-4 of the rhamnose moiety.[9,10] The side chains attached to position O-4 of the rhamnose can be classified into two different types. Arabinogalactan type I (AG I) composed of a predominately linear β-(1→4)-galactan branched with single α-L-Ara*f* units at position O-3 of the galactosyl units.[11] Linear or branched arabinans composed of α-L-(1,5)-L-Ara*f* units branched at position O-3 or O-2 with 1,2 and/or 1,3-linked α-L-arabinosyl side chains.[12] Apart from branching at position O-4 at the rhamnose unit, Renard et al.[13] have shown that in sugar beet ~2% of the Gal*p*A units bear a single β-D-Glc*p*A unit at position O-3.

Arabinogalactan type II (AG II), mainly associated with proteins (arabinogalactan protein AGP), is often co-extracted within RGI or pectic material. Until now no direct evidence has been found to whether this structural entity is covalently linked or associated with the pectin molecule,[14] and in references therein. AG II is the most predominant structure (>90%) within AGPs. AG II is composed of a β-(1→3)-galactan backbone branched at O-6 with short β-(1→6)-Gal*p* side chains terminated by α-L-Ara*f*. The side chains of AG II are often substituted by α-L-Ara*f*-(1→3) moieties, but other sugars such as Glc, Rha, and GlcA can also be found.[12,11]

RG II is found embedded in the HG chain and consists of a backbone of 8–10 GalA units.[15] The backbone of RG II is branched by complex side chains consisting of 12 different sugars and 20

* Corresponding author. Tel.: +31 317 482239; fax: +31 317 484893.
E-mail address: Henk.Schols@wur.nl (H.A. Schols).

0008-6215/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2008.09.030

different linkages.[16] The RG II structure is well conserved and hardly differs among plants of different species.[17]

All these different cell wall components present within MHR samples can be very effectively analyzed *in planta* by the use of different monoclonal antibodies (MAbs).[18] Within this paper, the use of such MAbs to test the distribution of different epitopes within MHR samples from different sources will be discussed. Therefore, analytical scale separations based on Mw and charge were performed and fractions were collected. The collected fractions were tested for the presence of different pectic epitopes.

2. Materials and methods

2.1. Substrates

The different substrates used within this study were obtained by enzymatic liquefaction of apple (MHRα and MHRB), carrot (MHRC), and potato (MHRP) according to Schols and Voragen.[19] All MHR samples were prepared using an experimental enzyme preparation called Rapidase C600 except for MHRB which was produced using Rapidase liq$^+$. Both enzymes were obtained from DSM Food Specialties, Delft, the Netherlands.

2.2. Monoclonal antibodies (MAbs)

Different MAbs were used to detect pectic structures after chromatographic separation. Arabinogalactan type I (AG I) was detected using LM5,[20] arabinan side chains were detected using LM6,[21] Arabinogalactan type II (AG II) was detected using LM2,[22] and homogalacturonan (HG) was detected using JIM 7 toward partially methyl esterified HG.[23]

2.3. Analytical methods

2.3.1. Sugar composition

Sugar compositions were determined using methanolysis followed by TFA hydrolysis according to de Ruiter et al.,[24] and were analyzed on a Dionex HPAEC system according to Verhoef et al.[25]

2.4. Linkage analyses

Linkage analyses were performed according to the Hakomori procedure.[26] Prior to permethylation of the different MHR samples, their uronic acid moieties were converted to their neutral sugar analogue and subsequently labeled at C-6 by deuterium according to Taylor and Conrad[27] modified by Kim and Carpita and Sims and Basic.[28,29]

The partly methylated alditol acetates were further analyzed by GC-FID and GC-MS according to Verhoef et al.[25]

2.5. Weak anion exchange chromatography

MHR samples (250 μL; 5 mg/mL) were separated and fractionated on a Dionex Propac™ WAX-10 column (250 × 4 mm) according to Guillotin et al.[30] using a Akta Purifier (GE Healthcare). Separation was monitored using UV 210 nm absorption, and fractions of 0.5 mL were collected in 96-well plates. The fractions were subsequently pooled for sugar composition analysis or further analyzed using ELISA detection with MAbs against different pectic epitopes.

2.6. HPSEC

High performance size exclusion chromatography was performed on a ThermoQuest HPLC using a series of three TosoHaas TSK-Gel G columns (4000PWXL, 3000PWXL, 2500PWXL). Samples (100 μL; 5 mg/mL) were separated at 30 °C using a 50 mM ammonium formiate buffer (pH 5.1) at 0.8 mL/min as eluent. Separation was monitored using a refractive index (RI) detector, and 0.5 mL fractions were collected in 96-well plates. The fractions were subsequently pooled for sugar composition analysis or further analyzed using MAb assays.

2.7. Source 15Q separation

MHRB was subjected to preparative separation based on their charge distribution using a Source 15Q column (115 × 60 mm, 325 mL). MHRB (0.5 g) in 100 mL was applied to the column at 25 mL/min. The sample was eluted off the column at 50 mL/min using a linear gradient from 0 to 600 mM phosphate buffer, pH 6. The gradient was applied using the following steps: 0–120 mM in 13 column volumes (CVs); 120–420 mM in 44 CVs; 420–600 mM in 2 CVs. Finally the column was washed with 8 CVs 600 mM phosphate buffer followed by 1 M NaOH (5 CVs). Fractions of 250 mL were collected using a SuperFrac fraction collector (GE healthcare). The elution profile was visualized using both UV 210 nm absorption and the neutral and uronic acid content of each fraction. Both neutral sugars and uronic acid were measured simultaneously using an automated colorimetric orcinol[31] and *m*-hydroxydiphenyl assay,[32,33] respectively.

2.8. ELISA assays

Enzyme-Linked Immuno Sorbent Assay (ELISA) was used to detect the different pectic structures present within each of the fractions. An aliquot of each fraction (20 μL) was diluted 10× using 0.01 M Phosphate Buffer Saline (PBS), pH 7.4, in an immunosorb 96-well plate (Maxisorb, F96, NUNC). The diluted fractions were allowed to coat to the plates overnight at 4 °C. After coating the plates were rinsed with tap water followed by blocking for 1 h at 4 °C using 200 μL PBS containing 3% (w/v) nonfat bovine milk powder (Sigma). After exhaustive washing with tap water, 100 μL primary antibody (10× diluted in PBS) was added and incubated for 2 h at room temperature. Again the plates were washed, and 100 μL of anti-rat IgG coupled to horseradish peroxidase (HRP, Sigma), 1000-fold dilution in PBS containing 1% (w/v) milk powder, was added and incubated for 1 h followed by another washing step.

The plates were developed by adding 150 μL 3,3′,5,5′-tetramethylbenzidine liquid substrate (Sigma). The reaction was stopped by adding 30 μL of 2 M H_2SO_4, resulting in the formation of a yellow color measured at 450 nm with a μQuant Microbiology ELISA reader.

2.9. Radial Yariv gel diffusion

Detection of AGII was performed by applying 8 μL MHR solution (3 mg/mL) to an 1% agarose gel with 0.15 M NaCl and 0.03 g/L Yariv phenylglycoside according to van Holst and Clarke.[34] After leaving the gel with the samples overnight the formed halo was compared to a calibration series of gum Arabic ranging from 0.1 to 2.5 mg/mL. The Yariv phenylglycoside was prepared as described by Yariv et al.[35] Yariv phenylglycoside positive material was precipitated from MHRC according to Immerzeel et al.[14]

3. Results and discussion

3.1. Extraction and composition of MHR from different sources

MHR was obtained by enzymatic liquefaction of apple (MHRα), carrot (MHRC), and potato (MHRP) using Rapidase C600 and apple

(MHRB) using Rapidase liq$^+$ according to Schols and Voragen.[19] These substrates were chosen since the RGI structure of these different plant species is known to differ within their side chain architecture. Within Tables 1 and 2, the sugar and linkage compositions of the different MHRs have been summarized.

Apple MHR is known to be rich in branched arabinan side chains.[36] The difference in MHRα and B is mainly caused by the enzyme preparation used for the liquefaction process. MHRα was obtained by a relatively mild liquefaction process using Rapidase C600, leaving the arabinans largely intact as reflected by the arabinose content of 25 mol % and the high amounts of α-(1,5)-linked Ara*f* together with α-(1,2), (1,3), (1,2,5), and (1,3,5)-linked Ara*f* moieties found after linkage analysis. The arabinose and α-(1,5)-linked Ara*f* content found for MHRB were much lower compared to MHRα, indicating that liquefaction with Rapidase liq$^+$, containing arabinanase activity, drastically shortens the arabinan side chains. As reflected in the increase in galactose and xylose contents within MHRB, degradation of these arabinans seems to result in a relative increase in XGA and AGII.

Although not all xylose and galacturonic acid have been recovered, the presence of XGA is proven by the increased amount of T-Xyl*p* and α-(1,3,4) and (1,2,4)-linked GalA*p* found within MHRB. The low yield of both GalA and Xyl within the linkage analysis could have been caused by either incomplete reduction of GalA or due to incomplete hydrolysis. Sugar composition analysis of the carboxyl-reduced sample of MHRB has shown that only 73% of the GalA was converted to galactose. Significant amounts of XGA have been found within apple before by Schols et al.[5] Compared to MHRα, MHRB contains much more β-(1,3), (1,6), and (1,3,6)-linked Gal*p*, T-Rha*p*, T-GlcA*p*, and T-Ara*f*. All these sugars are typically found within AGII.[12,11]

Carrot MHR was prepared since its extracted pectin is often found to be rich in AG II.[14,19,37] Furthermore, several studies have shown that AGII is an important structural feature for the bioactive properties of pectic extracts obtained from different plants.[38,39] Similar to MHRB, MHR isolated from carrot shows a high galactose content. Linkage analysis revealed that most of the galactose was found to be β-(1,3), (1,6), and (1,3,6)-linked. Furthermore, significant amounts of T-Rha*p*, T-GlcA*p*, and T-Ara*f* were found indicating presence of AGII. Both α-(1,5)-linked Ara*f* and β-(1,4)-linked Gal*p* were also found within MHRC, indicating that also arabinans and AGI are present as side chains.

Potato MHR is known to contain predominantly AGI side chains.[40,41] The MHR sample obtained from potato has a large proportion of galactose. This galactose was found to be β-(1,4)-linked, indicating the presence of AGI. Furthermore, the arabinose present was found to be predominately α-(1,5)-linked, indicating the presence of rather linear arabinans. The HG content of potato MHR is rather high as shown by the GalA (42 mol %) content. Together with a high GalA content, both the DM and DA are rather high in MHRP.

In general, the sugar compositions and especially the glycosidic linkage composition of these samples indicate that all of them show the presence of different pectic structures.

Table 1
Sugar composition (mol %) of MHR from different sources

	MHRα	MHRB	MHRC	MHRP
Fuc	1	0	2	1
Rha	15	11	22	10
Ara	25	11	8	14
Gal	15	20	32	28
Glc	1	3	2	4
Xyl	12	18	2	—[a]
GalA	31	37	30	42
GlcA	—	—	2	—
O-Me[b]	37	34	16	57
O-Ac	45	11	46	59
Rha:GalA	0.49	0.3	0.75	0.24
Total sugar (wt/wt %)	86	78	82	70

[a] — = not detected.
[b] *O*-Me: Moles MeOH per 100 moles GalA (max 100%), *O*-Ac: Moles Ac per 100 moles GalA (max 200%).

Table 2
Glycosidic linkage composition (mol %) of MHR samples from apple (MHRα and B), carrot (MHRC), and potato (MHRP)

	MHRα	MHRB	MHRC	MHRP
T[a]-Rha-*p*	0.9	1.2	1.1	0.3
1,2-Rha-*p*	9.7	5.0	8.9	4.6
1,4-Rha-*p*	1.2	2.1	2.0	1.3
1,2,4-Rha-*p*	11.7	17.1	16.3	13.4
	23.5	25.4	28.2	19.6
T-Ara-p	—[a]	0.8	0.2	0.1
T-Ara-*f*	3.8	4.0	2.5	1.6
1,2-Ara-*f*	0.2	0.5	0.1	—
1,5-Ara-*f*	20.0	2.8	1.5	18.2
1,3-Ara-*f*	2.3	1.2	0.1	—
1,3,5-Ara-*f*	1.8	0.3	—	—
1,2,5-Ara-*f*	1.9	0.9	—	—
1,2,3,5-Ara-*f*	1.8	1.2	0.4	0.6
	31.8	11.6	4.8	20.6
T-Gal-*p*	15.9	23.4	21.1	15.8
1,6-Gal-*p*	1.7	4.3	5.3	0.9
1,4-Gal-*p*	2.2	3.5	8.6	15.1
1,3-Gal-*p*	2.3	3.8	2.6	3.8
1,4,6-Gal-*p*	—	—	0.4	0.5
1,3,6-Gal-*p*	1.3	3.6	3.1	0.2
1,3,4,6-Gal-*p*	—	—	0.5	—
1,2,3,4,6-Gal-*p*	2.9	1.4	1.8	0.6
	26.3	40.0	43.4	36.9
T-Xyl-*p*	0.5	1.3	0.5	—
1,4-Xyl-*p*	0.9	1.1	—	0.4
1,2,4-Xyl-*p*	1.0	—	—	—
	2.4	2.4	0.5	0.4
T-Fuc-*p*	0.2	0.7	0.4	0.1
T-Glc-*p*	1.2	0.5	—	1.5
1,4-Glc-*p*	1.6	2.5	2.3	6.8
	2.8	3.0	2.3	8.3
1,4-GalA-*p*	10.3	12.2	12.8	12.2
1,2,4-GalA-*p*	0.5	0.9	0.9	0.3
1,3,4-GalA-*p*	0.5	1.2	2.3	0.8
	11.3	14.3	16.0	13.4
T-GlcA-*p*	1.2	0.8	4.1	—
1,4-GlcA-*p*	0.5	0.9	0.2	—
	1.7	1.7	4.3	—

[a] T = terminal; — = not detected.

3.2. Molecular weight and charge distribution of MHR from different sources

The four MHR samples obtained were separated by HPSEC chromatography to obtain more insight into the characteristics of the samples with respect to their molecular weight distribution. From the HPSEC elution profiles represented in Figure 1a it is obvious that all the samples are composed of three or more populations differing in their average molecular weight. These results show the heterogeneity of these samples with respect to their molecular weight especially for MHRB containing four major populations.

Furthermore, the samples were analyzed for their charge distribution using weak anion exchange chromatography. Figure 1b shows the WAX elution profiles of the MHR samples. It has to be

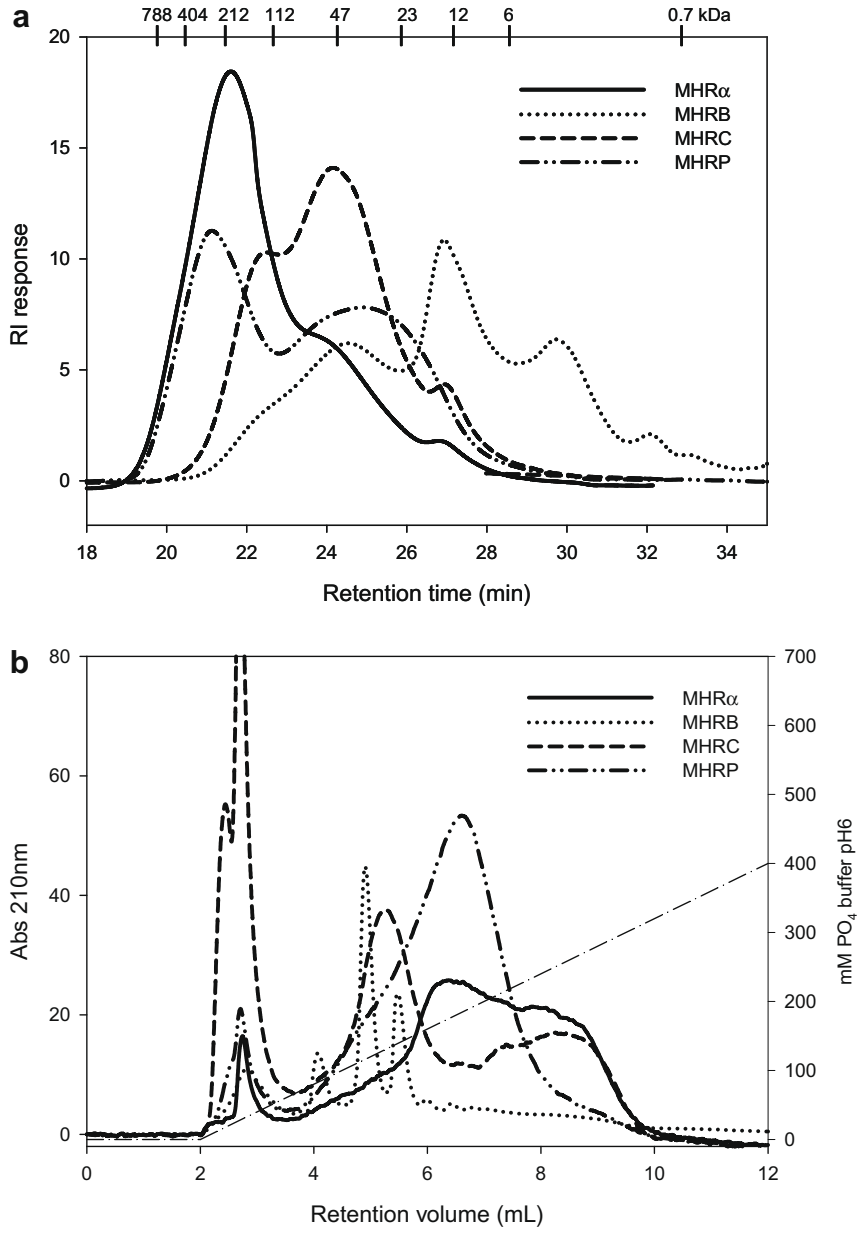

Figure 1. (a) HPSEC elution profiles of MHRα, MHRB, MHRC, and MHRP including molecular weight markers based on pullulan standards (top *x*-axis). (b) WAX HPLC elution profiles of MHRα, MHRB, MHRC, and MHRP and PO_4-buffer gradient.

noted that absorption at 210 nm was used for detection and therefore is only based on the galacturonic acid residues present. Due to this, the response is not proportional to the material present. The elution profiles show the presence of several populations for all the samples. Most of the samples show one neutral population and up to five retained populations. These results underline that also based on their charge several populations are present. Separation of industrial pectins based on the distribution of their methyl esterified carboxylic acid groups on a Dionex Propac, WAX-10 column has been proven to be successful.[30] Based on the results found for the methyl esterification of industrial pectins, we hypothesized that the natural length and distribution of the side chains in combination with the GalA present within MHR samples could largely influence their elution behavior on the same column. For example, treatment of RGI rich in arabinans with arabinanases causes the sample to interact more with the column, indicating that arabinans hinder the charge to interact with the column.[42]

3.3. HPLC followed by ELISA detection

To determine the epitopes responsible for the bioactive properties of, for example, MHRB, it is important to obtain more insight on the heterogeneity of these complex pectin samples. As indicated by the sugar/linkage composition and the size and charge characteristics of the samples, it is obvious that MHR consists of several pectic structures. Due to the presence of several molecular weight and charge populations, it was hypothesized that individual pectic structures could elute within separate populations, indicating the individual side chains to be attached to the RGI backbone in a cluster-like fashion (as suggested by Vincken et al.,[43] Fig. 2D).

Obtaining separate populations as observed within the HPSEC and WAX elution profiles followed by detailed analysis is obviously a time-consuming business. Therefore it was decided to combine ELISA assays using MAbs against different pectic epitopes with analytical HPSEC and WAX analyses of the different MHR samples.

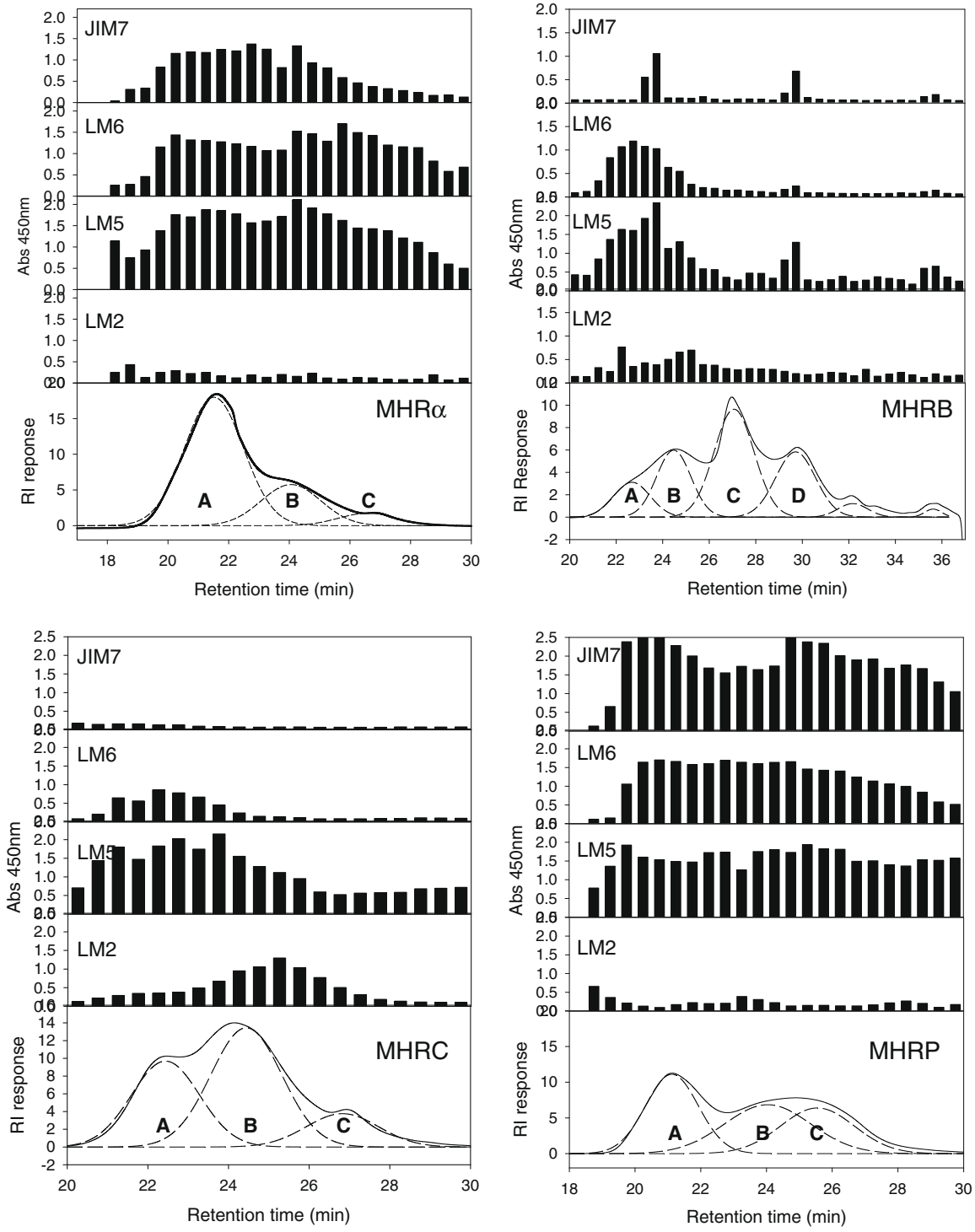

Figure 2. HPSEC elution profiles of MHRα, MHRB, MHRC, and MHRP combined with the ELISA response for the fractions collected. MAb LM2 was used for detection of AGII, LM5 for AGI, LM6 for arabinans, and JIM7 for HG.

This would provide a method for the fast fingerprinting of these pectic samples. The method was developed based on de Ruiter et al.[44] who have shown that the combination of HPSEC with ELISA detections to be successful for exopolysaccharides from *Mucorales*. For both separations, a maximum of 20 antibodies could be tested on one single analytical separation.

3.3.1. HPSEC followed by ELISA detection

The different MHR samples were first separated based on their molecular weight distribution using HPSEC. Fractions were obtained and tested using ELISAs with MAbs against different pectic epitopes. Figure 2 shows the HPSEC elution profiles of the different MHR samples together with the response of the different antibodies used to the collected fractions.

To verify the results obtained using different antibodies, the same HPSEC separation was performed to obtain the different populations, and subject these to sugar composition analysis. The sugar compositions of the different pools, as indicated in Figure 2, are shown in Table 3.

Table 3
Sugar composition (mol %) of pooled fractions (see Fig. 2) after HPSEC separation

	Fuc	Ara	Rha	Gal	Glc	Xyl	GalA	GlcA	Rha/GalA
MHRB									
A	2	7	21	23	4	17	26	0	0.82
B	3	6	19	22	2	16	31	2	0.62
C	3	9	19	16	1	11	39	2	0.5
D	1	7	18	14	2	14	44	1	0.4
MHRC									
A	0	5	25	39	2	2	23	4	1.11
B	2	5	25	35	1	2	28	2	0.88
C	3	7	24	27	2	1	34	2	0.7
MHRP									
A	0	18	16	47	2	1	17	0	0.9
B	0	15	14	34	4	1	32	0	0.44
C	1	6	7	12	7	0	65	2	0.11
MHRα									
A	1	24	17	13	2	14	29	1	0.57
B	1	21	19	17	1	11	29	2	0.66
C	3	17	15	12	4	7	39	3	0.38

For both MHRα and MHRP, no or only slight binding of LM2 was observed, reflecting the low AGII or AGP content within both these samples. Both these samples show significant epitope recognition by LM5, LM6, and JIM7 responsible for recognition of AGI, arabinan, and partially methyl esterified HG, respectively. The LM5 and LM6 response profiles more or less follow the RI pattern indicating that with respect to their side chain architecture all three populations observed are rather similar with respect to their structure. The same can be seen from the sugar compositions obtained from the individual populations.

For MHRα, the arabinose and galactose contents of pool A, B, and C are rather constant especially when the data are normalized based on the rhamnose content Rha:Ara and Rha:Gal ratios. From these ratios it is also evident that on average the arabinans are much longer than the galactans.

The galactose and arabinose contents of MHRP show a slight decrease from high to low molecular weight (A to C). These results indicate that over the three pools isolated from MHRP, the structure of the side chains does not seem to vary that much. On the other hand, pool C shows the presence of much more GalA probably derived from partially degraded HG during the liquefaction process.

For MHRB and MHRC, the picture is completely different. Separation of these samples by HPSEC causes antibody response to be restricted to only a number of populations.

For MHRB, at least four major populations can be observed, A, B, C, and D from HMw to LMw. Taking into account the overlap between the different populations, LM5, LM6, and JIM7 response is only observed for populations A and D.

Binding of LM5 and LM6 is predominantly observed for population A, indicating the presence of RGI material with both arabinan and AGI side chains. Hapten inhibition studies by Willats et al.[21] have shown that LM6 needs at least 5 to 6 arabinosyl units to be able to bind. This indicates that MHRB still contains short arabinan side chains with a minimum of five arabinose units, even though the Rapidase liq$^+$ enzyme preparation used to obtain MHRB has drastically shortened the arabinan side chains.

LM2 response is restricted to population B. From these results it is obvious that AGII elutes separately from AGI and arabinan. However, apart from the increased xylose content for populations A and B, the sugar compositions of the different pools are rather similar for all four populations. The increased xylose content for populations A and B indicates the presence of highly substituted xylogalacturonan within these two high molecular weight populations. The presence of XGA within the high molecular weight populations of apple MHR has been shown before by Schols et al.[5] Degradation by XGA hydrolase[45] did confirm the presence of XGA, although not all XGA could be degraded (results not shown). From the sum of the Rha:GalA and Xyl:Gala ratios, it is obvious that all the GalA present within these pools is either derived from RGI or from XGA. For pool A of both samples, the sum value of the ratios is above 1 indicating the presence of double xylosylated GalA units within the XGA present.

None of the antibodies show much response toward population C. Hilz et al.[46] have shown that RGII from bilberries, analyzed on the same column set and HPLC system, elutes at the same retention time as population C (approximately 27 min). The fact that none of the antibodies show response to this population and the slight increase in fucose and glucuronic acid (Table 3), sugars typical for RGII, could indicate that population C is enriched in RGII. Dedicated RGII analysis for this population should provide a more definite answer to the contents of population C. On the other hand, these results emphasize the need for an RGII-specific antibody.

For MHRC, AGI (LM5) and arabinan (LM6) were mainly observed in population A representing the HMw population. AGII (LM2) epitopes were found in population B.

No JIM7 response was observed for any of the three populations present correlating well with an Rha:GalA ratio found close to 1 for all the populations (Table 3). MHRC shows a rather similar sugar composition for populations A and B. Within population C, both the arabinose and galactose are slightly decreased, indicating that all three populations contain only slightly different pectic structures based on their sugar composition. Also for MHRC, none of the antibodies showed response toward population C eluting at the same retention time as the C population of MHRB.

In general, a rather constant subdivision in the elution behavior of MHR samples using HPSEC can be made. In the first population (A) between approximately 300 and 80 kDa RGI with arabinan and AGI sidechains elutes. The second population (B) between 80 and 30 kDa contains lower or higher levels of AGII depending on the source or the enzyme used for liquefaction. Even for MHRα or MHRP, slight recognition by LM2 is observed for this population. Population C eluting around 27 min (15 kDa) seems to be enriched in RGII. In the case of MHRB, population D mainly consists of low molecular weight degradation products of RGI and HG.

The results discussed above show that although the sugar compositions of the Mw populations present in a given MHR sample are rather similar, the nature of the side chains present could vary considerably. Especially for MHRC, it looks like AGII can be well separated from AGI and arabinans.

3.3.2. WAX HPLC combined with ELISA detection

Figure 3 shows the WAX elution profiles of the MHR samples together with the response of the different antibodies used for epitope detection. The elution profiles together with the antibody response show a clear separation between LM2 response and response to all other antibodies used. This clear separation is most obvious for MHRB and MHRC.

For MHRα and MHRP both containing only low AG II content, the low LM2 response is restricted to the less retained populations indicating that within these samples small quantities of AG II have been co-extracted.

As can be seen from the ELISA response profiles for MHRB and MHRC, a clear separation between AGII and both arabinan and AGI could be established using a WAX column. Analogous to MHRα and MHRP, AGII elutes first followed by AGI and arbinan.

The separation of LM2 epitope response from the other antibody responses clearly indicates that AGII is a separate entity within this pectic extract. What these results also demonstrate for certain is that AGII is certainly not mixed with the other side chains, and that it forms a separate building block of the pectic

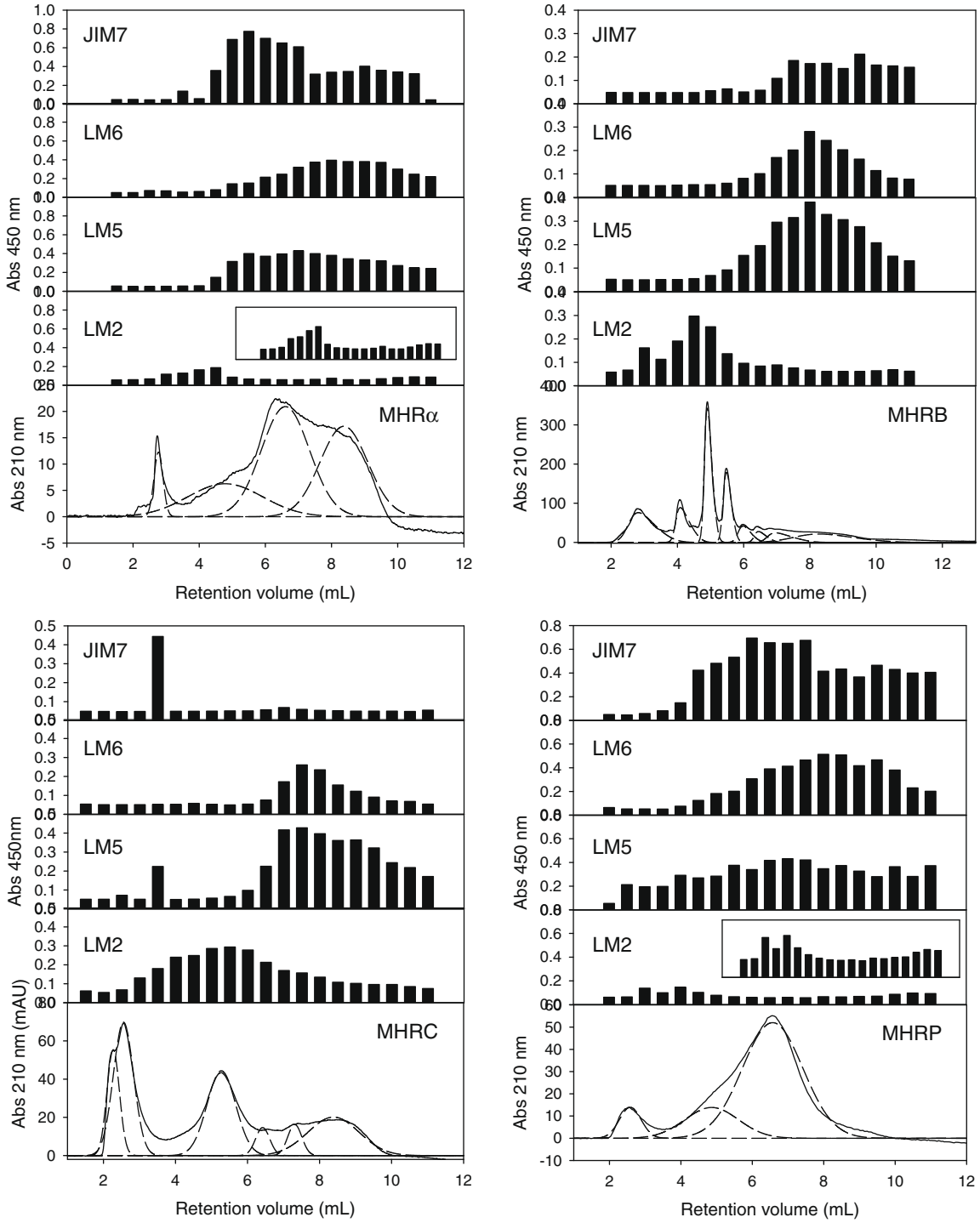

Figure 3. WAX HPLC elution profiles of MHRα, MHRB, MHRC, and MHRP combined with the ELISA response for the fractions collected. MAb LM2 was used for detection of AGII, LM5 for AGI, LM6 for arabinans, and JIM7 for HG.

molecule. The fact that the populations showing LM2 response are retained and that their sugar composition shows significant amounts of galacturonic acid and rhamnose, with a ratio close to 1, provides indirect proof for the linkage between AGII and pectin. The presence of such a linkage has been suggested by several authors,[12,14,43,47] although unambiguous evidence has never been found.

In general, separation of MHR samples using a WAX column provides us more distinction than HPSEC separation between different pectic structural entities. A drawback is that the UV detection used with this method is not proportional to the true amount of material eluting off the column.

3.4. Verification of WAX elution behavior by preparative separation of MHRB

Determination of the sugar composition upon analytical WAX separation appeared to be rather difficult. Both due to this and due to the clear separation observed between different epitopes, it was decided to subject MHRB to preparative scale separation.

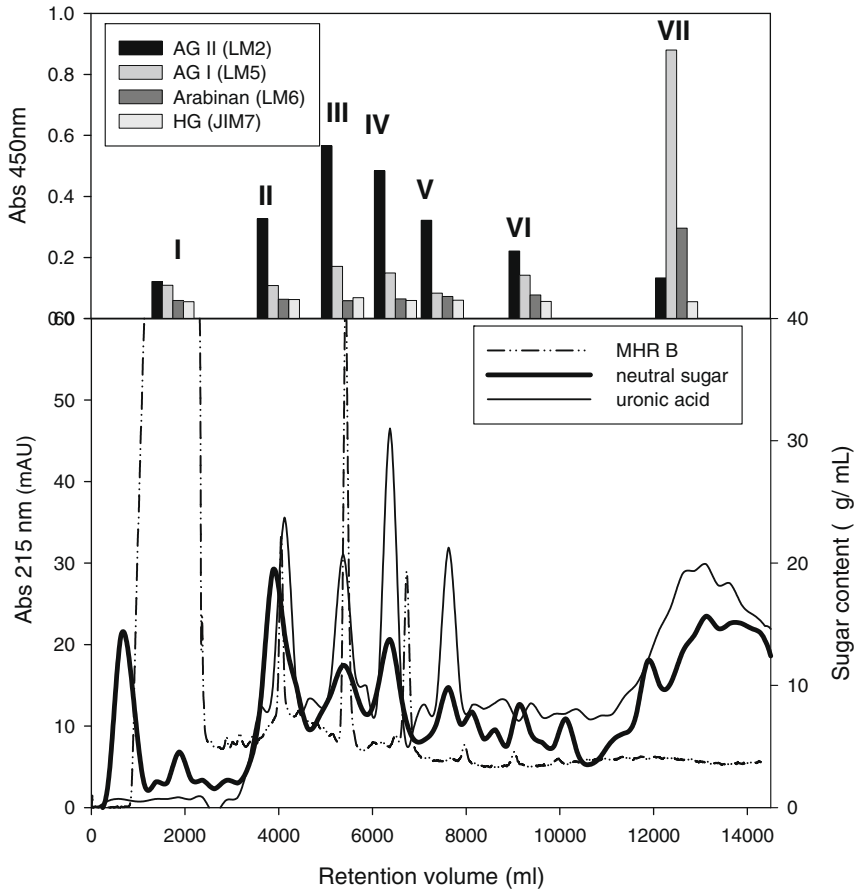

Figure 4. Bottom: Preparative Source 15Q elution profile of MHRB. The elution profiles were determined by UV 215 nm detection and measurement of the uronic acid and neutral sugar contents of the collected fractions. Top: ELISA response (abs 450 nm) of populations (in roman numerals) after pooling the fractions collected. MAb LM2 was used for detection of AGII, LM5 for AGI, LM6 for arabinans, and JIM7 for HG.

Therefore, enough material could be obtained to verify the conclusions based on the analytical scale separations followed by ELISA detection.

Since a Source 15Q column shows the same characteristics than the WAX-10 column, the separation of MHRB could be scaled up to obtain enough material of each of the different pools to perform more detailed structural analysis. The Source 15Q elution profile is represented in Figure 4. To compensate for the lack of UV absorbing groups within especially the neutral sugars for each of the collected fractions, the neutral sugar and uronic acid contents were determined. The elution profile is comparable to the results obtained with the WAX column as shown in Figure 1b. The fractions obtained were pooled, and are indicated as Roman numbers in Figure 4. Thereafter the different pools were subjected to HPSEC, WAX and sugar composition analyses.

3.4.1. HPSEC of Source 15Q pools

To be able to correlate the results obtained for the WAX column to the results obtained by HPSEC, the different AEC pools were analyzed by HPSEC. Figure 5 shows the HPSEC elution profiles of the different pools. In general, all the pools show the presence of more populations with respect to their molecular weight distribution. From pools I to VII, there is a gradual increase in molecular weight. Both pool VII and the PO_4 wash fraction show populations in the high molecular weight region (40–100 kDa), eluting at the same retention time as populations A, B, and C observed in the previous HPSEC runs (Figs. 1a, b and 2). Furthermore, the sharp intermediate molecular weight peak (approx. 27 min) seems to be exclusively present in pool VII.

These results indicate that based on charge, populations co-eluting with respect to their molecular weight distribution can be separated.

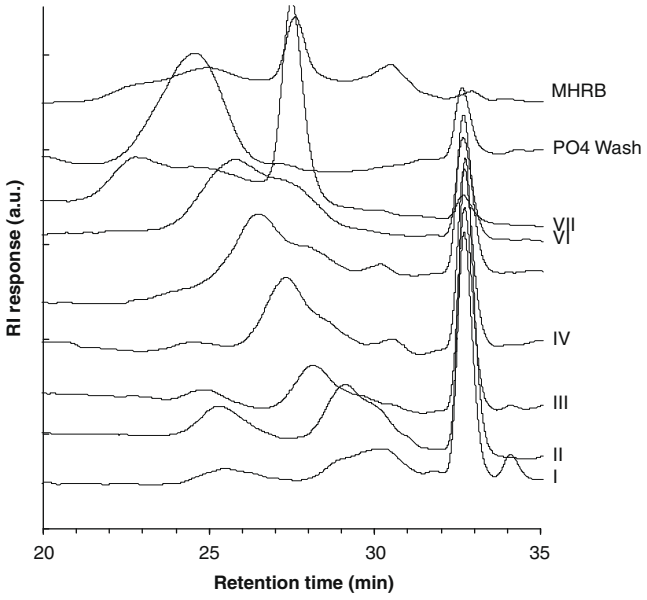

Figure 5. HPSEC elution profiles of the different pools obtained by Source 15Q separation of MHRB.

3.4.2. Sugar composition of Source 15Q pools

By determination of the sugar composition of the different pools obtained after the Source 15Q separation of MHRB, more information can be obtained of the different monomeric constituents present. Consequently, these data can then be translated to the different pectic polymers expected to be present and compared to the antibody response profiles found.

Table 4 shows the sugar composition of the different pools in mol %, furthermore, Table 4 shows the Rha:GalA and Xyl:GalA ratios.

It is obvious that the GalA content increases with an increase in retention time. Obviously a higher GalA content is directly correlated to the amount of charged groups able to interact with the column. Apart from pool VII, xylose shows an increase parallel to the GalA content. MHR B is known to be enriched in xylogalacturonan and therefore the xylose is most probably derived from this polymer. The Rha:GalA ratio of pools III to VII shows that approximately 40–50% of the GalA is derived from the RGI backbone. Similarly, the Xyl:GalA ratio of these fractions indicates that 40–50% of the GalA is derived from the xylogalacturonan backbone. For all pools the sum of both these ratios is close to 1, indicating that virtually no HG is present in MHRB. The certain drop in xylose content for pool VII possibly indicates that the XGA present within this pool is less xylosylated than within the other populations.

Table 4
Sugar composition of pooled fractions after Source 15Q separation of MHRB (mol %) including Rha:GalA and Xyl:Gala ratios

	I	II	III	IV	V	VI	VII	600 mM PO_4 wash
Fucose	1	1	1	1	1	1	3	3
Rhamnose	19	16	15	15	15	13	16	19
Arabinose	11	10	9	8	8	6	8	2
Galactose	37	37	31	25	24	17	13	9
Glucose	1	1	1	1	0	0	1	1
Xylose	2	6	14	18	19	26	14	19
GalA	27	26	29	31	32	36	44	46
GlcA	2	3	1	1	1	0	1	1
Rha:GalA	0.7	0.6	0.5	0.5	0.5	0.4	0.4	0.4
xyl:GalA	0.1	0.2	0.5	0.6	0.6	0.7	0.3	0.4
Sum	0.7	0.9	1.0	1.1	1.1	1.1	0.7	0.8

In contrast to the increase in GalA and Xyl, the Gal content shows a decrease with an increase in retention time. The arabinose content seems to be constant for all the pools. These two observations indicate that with an increase in retention time the AGII content decreases and the AGI and arabinan contents increase. Combining these results with the Mw distribution of the different pools, the first pools seem to contain rather long AG II polymers. In contrast, pool VII seems to contain RG I with short arabinan and AG I side chains. All the pools obtained contain low levels of GlcA, a sugar typically found at the terminus of the side chains present on the AGII backbone. The GlcA content for the first two pools seems to be slightly higher than for the other pools.

3.4.3. Detection of different pectic epitopes within the isolated pools by MAb detection

To be able to directly compare the results obtained after analytical WAX separation, the pools obtained by AEC were also subjected to ELISAs using MAbs against pectic epitopes (Fig. 4).

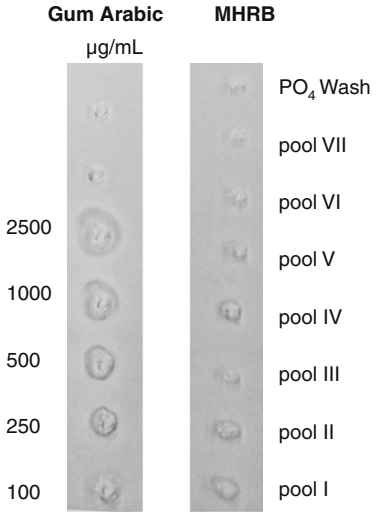

Figure 6. Yariv phenylglycoside radial diffusion gel of MHRB pool obtained after source 15Q separation and Gum Arabic calibration curve.

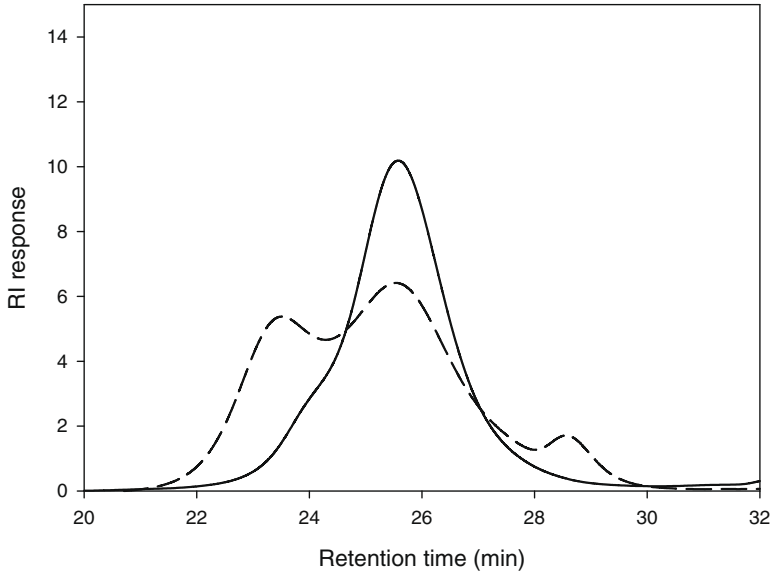

Figure 7. HPSEC elution profiles of MHRC separated in Yariv + or precipitated (—) and Yariv − or supernatant (**- - - -**) material using precipitation of AGII with Yariv phenylglycosides.

The low JIM7 (HG) response toward all the isolated pools further substantiates the observation that MHRB has a low HG content and that all GalA is either incorporated within the RGI backbone or within XGA. Furthermore, both LM5 (β-1,4-galactan) and LM6 (α-1,5-arabinan) antibodies were used. Both the antibodies show most recognition toward pool VII and the PO_4 pool indicating that RGI with arabinan and AGI side chains attached to it show strong interaction to the column. On the other hand pools II to VI seem to show recognition predominantly by LM2, indicating the presence of AGII within these pools. Again these results further substantiate the conclusions already made by the sugar composition of these pools.

3.4.4. Confirmation of AGII by Yariv radial gel diffusion and precipitation

Since LM2 is only diagnostic for one epitope present on AG II, Yariv radial gel diffusion was used to confirm the presence of AGII only in the LM2 positive populations of MHRB. Figure 6 shows the image of the developed Yariv gel for the pools obtained after Source 15Q separation. From the image it is evident that AG II is only present within the LM2-positive pools of MHRB, and no other AG II glycans are present eluting at different conditions.

Furthermore, the AG II present in MHR C was precipitated using Yariv phenyl glycosides. Figure 7 shows the HPSEC elution profiles of both the supernatant and the Yariv-positive material of MHRC. The elution profiles as represented in Figure 7 clearly illustrate that also after HPSEC separation no other AG II is present eluting at different retention times, since the Yariv precipitated material elutes at the same retention time as the LM2-positive pool shown in Figure 2.

4. Conclusions

HPSEC and WAX analyses combined with ELISA detection showed to be a successful method for fingerprinting complex pectin mixtures. Using a single analytical separation, the location of different pectic epitopes within the elution profile of a given sample could be established. This new approach provides a useful tool to obtain fast insight into the heterogeneity of different pectic extracts. Rather than first having to obtain substantial amounts of the different populations present and subjecting these to different biochemical characterization methods such as linkage and sugar composition analyses. Furthermore, the outcome of these analyses could be used for more targeted purification of predefined pectic polysaccharides. The method has been validated by means of preparative separation of the MHR samples and classical biochemical characterization of the same samples. The method can easily be extended using other or new antibodies. Attempts to find correlations between bioactivity and the responsible pectic epitope could, for example, be made by determination of fibronectin binding profiles to the same fractions using a sandwich ELISA approach as shown before to be successful on non-separated pectin samples coated to polystyrene surfaces by Nagel et al.[48] Fibronectin is a high molecular weight glycoprotein involved in the adhesion of mammalian cells to a given surface.[49]

In the future, attempts will be made to further automate the methods by further developing this method toward microarray analysis of the different fractions obtained using the method described by Moller et al.[50,51]

References

1. Kokkonen, H. E.; Ilvesaro, J. M.; Morra, M.; Schols, H. A.; Tuukkanen, J. *Biomacromolecules* **2007**, *8*, 509–515.
2. Morra, M.; Cassinelli, C.; Cascardo, G.; Nagel, M.-D.; Della Volpe, C.; Siboni, S.; Maniglio, D.; Brugnara, M.; Ceccone, G.; Schols, H. A. *Biomacromolecules* **2004**, *5*, 2094–2104.
3. Bussy, C.; Verhoef, R.; Haeger, A.; Morra, M.; Duval, J.-L.; Vigneron, P.; Bensoussan, A.; Velzenberger, E.; Cascardo, G.; Cassinelli, C.; Schols, H. A.; Knox, J. P.; Nagel, M.-D. *J. Biomed. Mater. Res. Part A.* **2008**, *86A*, 597–606.
4. Schols, H. A.; In 't Veld, P. H.; Vandeelen, W.; Voragen, A. G. J. *Z. Lebensm. Unters. Forsch.* **1991**, *192*, 142–148.
5. Schols, H. A.; Bakx, E. J.; Schipper, D.; Voragen, A. G. J. *Carbohydr. Res.* **1995**, *279*, 265–279.
6. Ishii, T. *Plant Physiol.* **1997**, *113*, 1265–1272.
7. Komalavilas, P.; Mort, A. J. *Carbohydr. Res.* **1989**, *189*, 261–272.
8. Lerouge, P.; Oneill, M. A.; Darvill, A. G.; Albersheim, P. *Carbohydr. Res.* **1993**, *243*, 359–371.
9. Lau, J. M.; McNeil, M.; Darvill, A. G.; Albersheim, P. *Carbohydr. Res.* **1985**, *137*, 111–125.
10. McNeil, M.; Darvill, A. G.; Albersheim, P. *Plant Physiol.* **1980**, *66*, 1128–1134.
11. Clarke, A. E.; Anderson, R. L.; Stone, B. A. *Phytochemistry* **1979**, *18*, 521–540.
12. Ridley, B. L.; O'Neill, M. A.; Mohnen, D. *Phytochemistry* **2001**, *57*, 929–967.
13. Renard, C.; Crepeau, M. J.; Thibault, J. F. *Eur. J. Biochem.* **1999**, *266*, 566–574.
14. Immerzeel, P.; Eppink, M. M.; de Vries, S. C.; Schols, H. A.; Voragen, A. G. J. *Physiol. Plant.* **2006**, *128*, 18–28.
15. Ishii, T.; Matsunaga, T. *Phytochemistry* **2001**, *57*, 969–974.
16. O'Neill, M. A.; Warrenfeltz, D.; Kates, K.; Pellerin, P.; Doco, T.; Darvill, A. G.; Albersheim, P. *J. Biol. Chem.* **1996**, *271*, 22923–22930.
17. O'Neill, M. A.; Ishii, T.; Albersheim, P.; Darvill, A. G. *Annu. Rev. Plant Biol.* **2004**, *55*, 109–139.
18. Knox, J. P. Cell and Developmental Biology of Pectins. In *Pectins and Their Manipulation*; Seymour, G. B., Knox, J. P., Eds.; Blackwell Publishing Ltd: Oxford, 2002.
19. Schols, H. A.; Voragen, A. G. J. *Carbohydr. Res.* **1994**, *256*, 83–95.
20. Jones, L.; Seymour, G. B.; Knox, J. P. *Plant Physiol.* **1997**, *113*, 1405–1412.
21. Willats, W. G. T.; Marcus, S. E.; Knox, J. P. *Carbohydr. Res.* **1998**, *308*, 149–152.
22. Yates, E. A.; Valdor, J. F.; Haslam, S. M.; Morris, H. R.; Dell, A.; Mackie, W.; Knox, J. P. *Glycobiology* **1996**, *6*, 131–139.
23. Knox, J. P.; Linstead, P. J.; King, J.; Cooper, C.; Roberts, K. *Planta* **1990**, *181*, 512–521.
24. de Ruiter, G. A.; Schols, H. A.; Voragen, A. G. J.; Rombouts, F. M. *Anal. Biochem.* **1992**, *207*, 176–185.
25. Verhoef, R.; de Waard, P.; Schols, H. A.; Rättö, M.; Siika-aho, M.; Voragen, A. G. J. *Carbohydr. Res.* **2002**, *337*, 1821–1831.
26. Hakomori, S. *J. Biochem.* **1964**, *55*, 205–208.
27. Taylor, R. L.; Conrad, H. E. *Biochemistry* **1972**, *11*, 1383.
28. Kim, J. B.; Carpita, N. C. *Plant Physiol.* **1992**, *98*, 646–653.
29. Sims, I. M.; Bacic, A. *Phytochemistry* **1995**, *38*, 1397–1405.
30. Guillotin, S. E.; Van Loey, A.; Boulenguer, P.; Schols, H. A.; Voragen, A. G. J. *Food Hydrocolloids* **2007**, *21*, 85–91.
31. Tollier, M. T.; Robin, J. P. *Ann. Technol. Agr.* **1979**, *28*, 1–15.
32. Ahmed, A. E. R.; Labavitch, J. M. *J. Food Biochem.* **1977**, *1*, 361–365.
33. Thibault, J. F. *Lebensm. Wis. Technol.* **1979**, *12*, 247–251.
34. van Holst, G. J.; Clarke, A. E. *Anal. Biochem.* **1985**, *148*, 446–450.
35. Yariv, J.; Rapport, M. M.; Graf, L. *Biochem. J.* **1962**, *85*, 383–388.
36. Schols, H. A.; Posthumus, M. A.; Voragen, A. G. J. *Carbohydr. Res.* **1990**, *206*, 117–129.
37. Immerzeel, P.; Schols, H. A.; Voragen, A. G. J.; de Vries, S. C. *Physiol. Plant.* **2004**, *122*, 181–189.
38. Paulsen, B. S.; Barsett, H. *Adv. Polym. Sci.* **2005**, *186*, 69–101.
39. Yamada, H.; Kiyohara, H. Immunomodulating Activity of Plant Polysaccharide Structures. In *Comprehensive Glycoscience From Chemistry to Systems Biology*; Boons, G.-J., Lee, Y. C., Suzuki, A., Taniguchi, N., Voragen, A. G. J., Eds.; Elsevier: Amsterdam, 2007; pp 663–694.
40. Sørensen, S. O.; Pauly, M.; Bush, M.; Skjøt, M.; McCann, M. C.; Borkhardt, B.; Ulvskov, P. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 7639–7644.
41. Wood, P. J.; Siddiqui, I. R. *Carbohydr. Res.* **1972**, *22*, 212.
42. Westereng, B. Pectin from cabbage (Brassica oleracea). Studies on Structure and Bioactivity. PHD-thesis Faculty of Mathematics and Natural Sciences, 2007, University of Oslo: Oslo.
43. Vincken, J.-P.; Schols, H. A.; Oomen, R. J. F. J.; McCann, M. C.; Ulvskov, P.; Voragen, A. G. J.; Visser, R. G. F. *Plant Physiol.* **2003**, *132*, 1781–1789.
44. de Ruiter, G. A.; van der Lugt, A. W.; Voragen, A. G. J.; Rombouts, F. M.; Notermans, S. H. W. *Carbohydr. Res.* **1991**, *215*, 47–57.
45. Zandleven, J.; Beldman, G.; Bosveld, M.; Benen, J.; Voragen, A. *Biochem. J.* **2005**, *387*, 719–725.
46. Hilz, H.; Williams, P.; Doco, T.; Schols, H. A.; Voragen, A. G. J. *Carbohydr. Polym.* **2006**, *65*, 521–528.
47. Scheller, H. V.; Jensen, J. K.; Sørensen, S. O.; Harholt, J.; Geshi, N. *Physiol. Plant.* **2007**, *129*, 283–295.
48. Nagel, M.-D.; Verhoef, R.; Schols, H.; Morra, M.; Knox, J. P.; Ceccone, G.; Della Volpe, C.; Vigneron, P.; Bussy, C.; Gallet, M.; Velzenberger, E.; Vayssade, M.; Cascardo, G.; Cassinelli, C.; Haeger, A.; Gilliland, D.; Liakos, I.; Rodriguez-Valverde, M.; Siboni, S. *Biochem. Biophys. Acta* **2008**, *1780*, 995–1003.
49. Yamada, K. M.; Olden, K. *Nature* **1978**, *275*, 179–184.
50. Moller, I.; Marcus, S. E.; Haeger, A.; Verhertbruggen, Y.; Verhoef, R.; Schols, H.; Ulvskov, P.; Mikkelsen, J. D.; Knox, J. P.; Willats, W. *Glycoconjugate J.* **2008**, *25*, 37–48.
51. Moller, I.; Sørensen, I.; Bernal, A. J.; Blaukopf, C.; Lee, K.; Øbro, J.; Pettolino, F.; Roberts, A.; Mikkelsen, J. D.; Knox, J. P.; Bacic, A.; Willats, W. G. T. *The Plant J.* **2007**, *50*, 1118–1128.