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High-performance liquid chromatographic separation and on-line mass spectrometric detection of saturated and unsaturated oligogalacturonic acids

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Abstract

A method for the simultaneous determination of saturated and unsaturated oligogalacturonic acids up to degree of polymerization (dp) of 7 by high-performance liquid chromatography (HPLC) is presented. For this purpose, a Cyclobond I 2000 column and a volatile mobile phase consisting of ammonium formate and methanol were used, allowing direct coupling of HPLC to a mass spectrometer via an electrospray interface (ESI-MS) without additional desalting. The analytical system was used for the characterization of digests obtained by incubation of polygalacturonic acid with commercial enzyme preparations.

Keywords: Oligogalacturonic acids; HPLC; Electrospray-ionization mass spectrometry; Enzyme activity

1. Introduction

Pectins are a group of complex polysaccharides found in the cell walls of higher plants. Oligogalacturonic acids (OGAs) released from the homogalacturonan-backbone (linear chain of (1→4)-linked α-D-galactopyranosyluronic acid) have been shown to exhibit a number of biological activities in plants such as induction of defense response and regulation of growth and development.[1] Depolymerization by glycosidases or acid hydrolysis leads to the formation of saturated oligomers, whereas 4,5-unsaturated OGAs are a result of the β-eliminative cleavage by lyases. The latter may also be formed non-enzymatically by lye treatment of fruits and vegetables and by thermal degradation of pectins under neutral or alkaline pH conditions.

Apart from their hormone-like function in plants, OGAs have recently attracted intense interest since they have been demonstrated to inhibit the adherence of bacteria to epithelial cells and might therefore be used as therapeutic agents.[2,3] However, the reports on the exact chemical structure of the biologically active principles are conflicting. Whereas OGAs with a degree of polymerization (dp) of 2–7 have been held responsible for the anti-adhesive effect,[2,3] more recent investigations have pointed out that the effect primarily depends on the presence of a terminal unsaturated uronic acid but not on the dp or the extent of esterification of the fragments.[4] Therefore, reliable analytical methods for the unambiguous characterization of pectin degradation products are urgently needed.

In the past, various analytical techniques such as thin-layer chromatography,[5] capillary electrophoresis[6] and high-performance liquid chromatography have been applied for the separation of OGAs. Among the HPLC methods, ion-pair reversed-phase,[7,8] ion-exchange and size-exclusion[9,10] chromatography have been described, high-performance anion-exchange chromatography (HPAEC) combined with pulsed amperometric detection being the most frequently used technique.[11,12] The simultaneous separation of saturated and unsaturated oligomers with pulsed amperometric[13,14] or diode array detection[15] has recently been reported. β-Cyclodextrin-bonded stationary

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phases have been successfully employed for the separation of neutral mono- and oligosaccharides,[16,17] as well as of saturated and unsaturated OGAs.[18]

However, the often unpredictable elution order of oligosaccharides, the instability of retention times, and the apparent lack of commercially available reference compounds are a considerable problem. Therefore, mass spectrometric detection has been increasingly employed for the characterization of OGAs. However, the incompatibility of sodium containing buffers with MS requires additional desalting steps which are tedious and time-consuming. Therefore, the main objective of the present study was to establish an analytical system for the separation of saturated and unsaturated OGAs which allowed their on-line mass spectrometric detection. Owing to their putative physiological relevance, particular attention was given to oligomers with a dp up to 7.

2. Results and discussion

The separation of a standard mixture of saturated and unsaturated OGAs is shown in Fig. 1(a). As can be seen, baseline separation up to dp 7 (saturated) was achieved within 40 min using the isocratic eluent system 1. While UV detection at 235 nm allowed the specific determination of unsaturated compounds (Fig. 1b), both saturated and unsaturated OGAs could be monitored by RI detection. Thus, in contrast to pulsed amperometric detection, immediate differentiation of saturated and unsaturated analytes was possible.

Since strong peak tailing, especially of the late-eluting compounds, and a loss of sensitivity were observed as a consequence of isocratic elution, the method was further improved by using an eluent with higher ionic strength (eluent system 2) and employing mass spectrometric detection for the characterization of OGAs. A series of chromatograms obtained by extraction of the m/z values of the singly charged ions of OGAs of dp 1–5 $[M-H]^-$, and of doubly charged ions $[M-2H]^{2-}$ of dp 6–8 are shown in Figs. 2(a) and (b), respectively. Due to the ionization conditions applied, only singly and doubly charged ions without further fragmentation were obtained. Singly charged ions were observed up to dp 5, whereas higher-molecular OGAs presumably produced multiply charged molecular ions. The use of a volatile buffer of a higher ionic strength allowed accelerated elution and reduced total analysis time to approximately 15 min.

The analytical system was applied to the determination of pectolytic activities of several commercial enzyme preparations using eluent 1. All enzymes investigated displayed polygalacturonase activity, whereas lyase activity could not be observed under the conditions applied. As shown in Table 1, saturated OGAs up to dp 3 were generally formed after 2 h of incubation, whereas higher-molecular fragments could only be detected in exceptional cases. The contents of OGAs were calculated as monogalacturonic acid from the RI signals since homooligosaccharides were shown to yield reasonably equivalent peak areas.[18] In addition to the pectolytic enzymes listed in Table 1, Rohalase 7069 which was specified as an exclusively cellulolytic enzyme preparation was investigated for pectolytic side activities. The OGA profile obtained after incubation of polygalacturonic acid with Rohalase 7069 is shown in Fig. 3. As demonstrated by RI detection, saturated OGAs with dp 2–14 were released by polygalacturonase, thus indicating considerable pectolytic side activities.

Owing to their various biological activities, OGAs have gained increasing interest in the past years. However, detailed studies have often been hampered by limitations of analytical methods that have not allowed the unambiguous characterization of biologically active compounds. Therefore, mass spectrometry has become the detection of choice since it provides rapid and extensive information on the chemical nature of OGAs.[19–21] The main problem of mass spectrometry consists in the fact that desalting is required to avoid soiling of the ion source and loss of sensitivity. Considerable progress has been made by the introduction of on-line desalting devices.[22–24]

The use of volatile buffers represents an alternative for on-line coupling of HPLC and mass spectrometry. In the present study this approach has been successfully applied to the simultaneous determination of saturated and unsaturated OGAs and to the characterization of pectolytic activities and side activities, respectively, of a number of commercial enzyme preparations. It is expected that the method established might also be useful for kinetic studies on pectin degradation and for the detection of an extensive use of pectolytic enzymes in fruit and vegetable juice production.[25] Furthermore, the effects of thermal treatment of pectin-containing preparations in acidic or neutral milieu may be investigated using this analytical system.

3. Experimental

Materials and reagents.—Reagents and solvents used were of analytical or HPLC grade (E. Merck, Darmstadt, Germany). Ammonium formate and galacturonic acid were purchased from Fluka (Buchs, Switzerland). Polygalacturonic acid (Na salt) was obtained from Serva (Heidelberg, Germany). Polygalacturonic acid solution (1%, w/w) was prepared as follows: A 50 mM solution of ammonium formate (pH 5) was heated at 70 °C for 5 min. PGA (500 mg) was added under stirring. After 15 min the pH was adjusted to 5 and 8.5

with diluted formic acid and diluted NaOH prior to incubation with polygalacturonase and pectate lyase, respectively. *endo*-Polygalacturonase (EC 3.2.1.15) from *Aspergillus japonicus* was obtained from Sigma (St. Louis, MO, USA). The enzyme preparations pectate lyase, Pectinex Ultra SP-L, Pectinex Smash, Pectinex BE 3L, and Viscozyme L were a gift from Novo Nordisk Ferment (Dittingen, Switzerland). Rohalase 7069, Rohament PL, Rohapect MA Plus, Rohapect B5L, and Rohapect B1L were kindly provided by Röhm Enzyme (Darmstadt, Germany).

Preparation of standard solutions of saturated and unsaturated OGAs.—A mixture of saturated oligogalacturonic acids was prepared by addition of 7 μL (2 units) of *endo*-polygalacturonase to polygalacturonic acid solution (pH 5). After stirring at 40 °C for 3 h, the

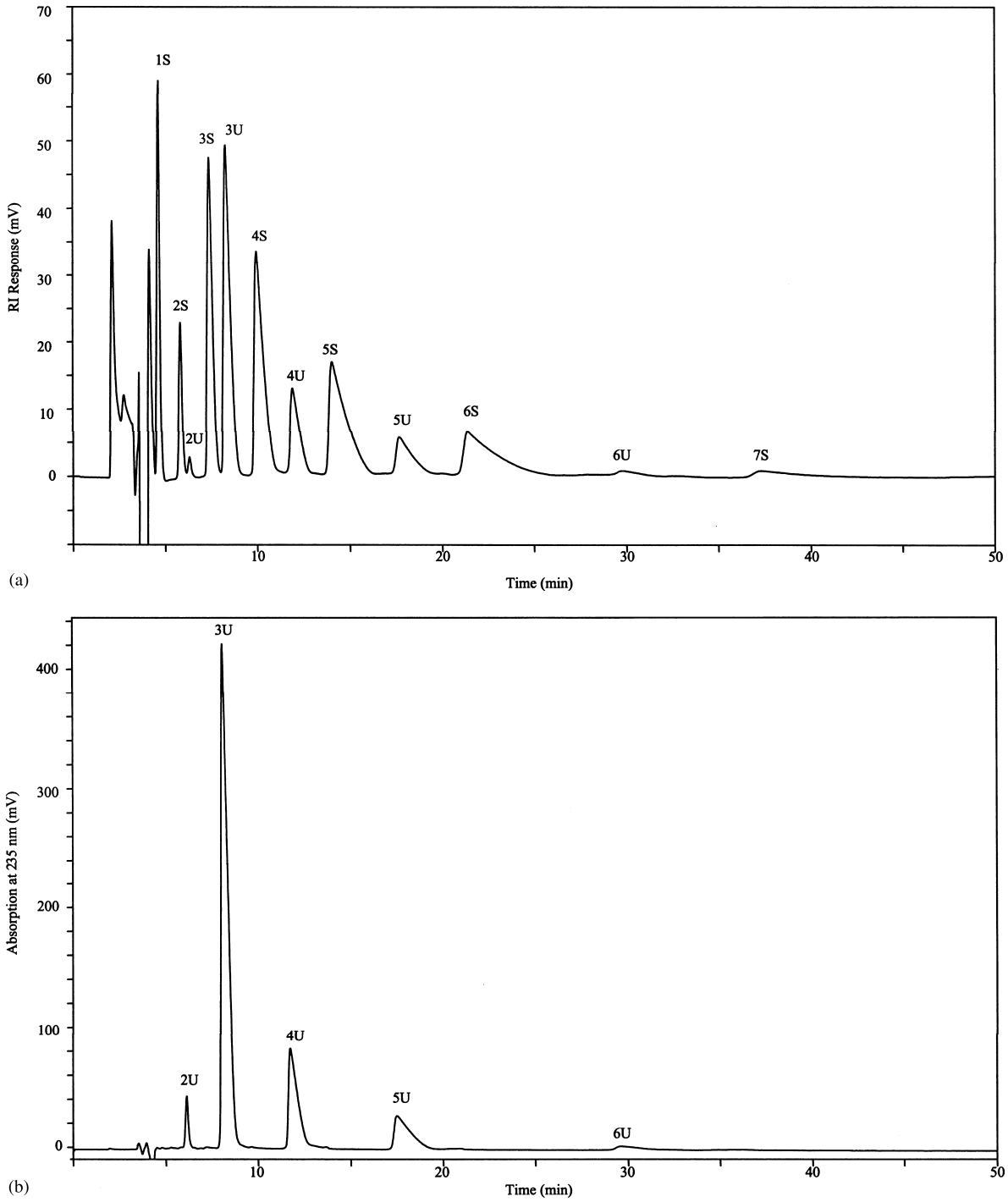

Fig. 1. Separation of a standard mixture of saturated (S) and unsaturated (U) OGAs and monogalacturonic acid (peak numbers indicate degree of polymerization) with (a) RI detection and (b) UV detection (235 nm) using eluent system 1.

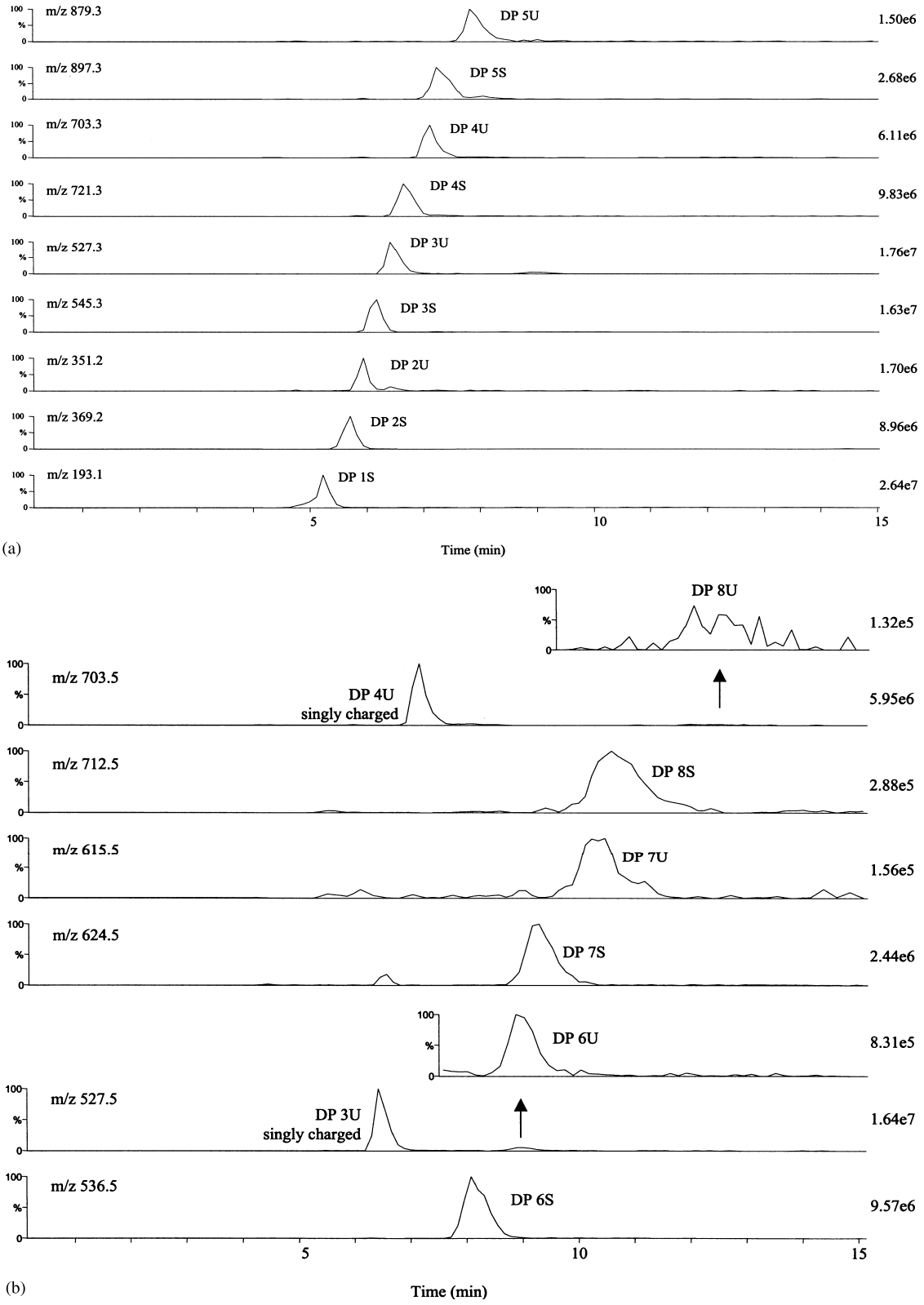

Fig. 2. LC–MS chromatograms of saturated and unsaturated OGAs (m/z values extracted from full-scan aquisition). (a) Singly charged ions $[M-H]^-$ for dp 1–5, and (b) doubly charged ions $[M-2H]^{2-}$ for dp 6–8. Eluent system 2 was used for the separation of OGAs.

Table 1
Contents (g/L, calculated as monogalacturonic acid) of saturated OGAs obtained after 2 h incubation of polygalacturonic acid with commercial pectolytic enzyme preparations (eluent system 1, RI detection)

Enzyme preparation	Degree of polymerization[a]							Total
	1	2	3	4	5	6	7	
Pectinex BE 3L	0.69	0.58	0.76	1.03	1.55	0.46	0.14	5.21
Rohapect B1L	0.59	0.92	1.51	2.21	0.42	–	–	5.65
Rohapect B5L	0.52	0.90	1.37	2.18	0.65	–	–	5.63
Rohament PL	1.04	0.97	2.85	0.70	–	–	–	5.55
Pectinex Smash	1.03	1.20	2.30	1.04	–	–	–	5.57
Pectinex Ultra SP-L	1.23	1.34	2.28	0.66	–	–	–	5.51
Viscozyme L	1.47	1.40	2.30	0.45	–	–	–	5.62
Rohapect MA Plus	0.55	2.10	3.09	–	–	–	–	5.73

[a] –, not detectable.

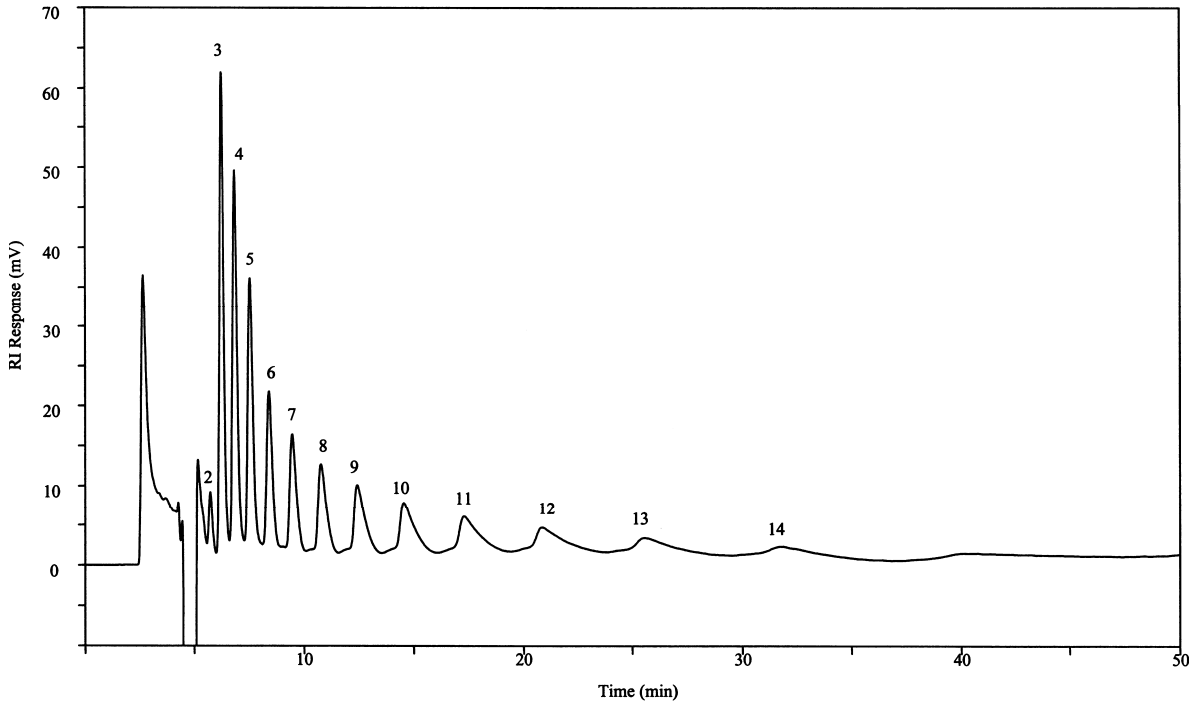

Fig. 3. Separation of OGAs (peak numbers indicate degree of polymerization) released from polygalacturonic acid after incubation with the commercial cellulase preparation Rohalase 7069 (eluent system 2, RI detection).

reaction was stopped by heating at 90–95 °C for 10 min, followed by rapid cooling in an ice bath. Unsaturated oligogalacturonic acids were generated by addition of 10 μL of pectate lyase preparation to the polygalacturonic acid solution (pH 8.5, 40 °C). After 2 h the solution was treated as described above. A standard solution of saturated and unsaturated OGAs was prepared by mixing the polygalacturonic acid digests with monogalacturonic acid solution (5 g/L) at a ratio of 6:3:1 (v/v/v). All samples were filtered (0.2 μm) prior to HPLC analysis.

Sample preparation for the determination of enzyme activities.—Aliquots of 10 μL of each of the enzyme preparations Pectinex Ultra SP-L (23496 ppm), Pectinex Smash (23386 ppm), Pectinex BE 3L (23350 ppm), Viscozyme L (24270 ppm), Rohalase 7069 (23486 ppm), Rohament PL (23578 ppm), Rohapect MA Plus (23642 ppm), Rohapect B5L (23788 ppm) and Rohapect B1L (23914 ppm) were added separately to the polygalacturonic acid solution (pH 5) at 40 °C. The reaction was stopped after 2 h by heating at 90–95 °C for 10 min. After cooling in an ice bath, an aliquot was filtered (0.2 μm) and used for HPLC analysis.

Quantification of saturated OGAs.—The quantification of mono- and oligogalacturonic acids was based on a six-point external standard curve. Five standards

ranging from 0.046 to 2.745 g/L were prepared by diluting a stock solution of monogalacturonic acid (4.576 g/L). Standards and stock solution were injected twice. Concentrations of saturated oligomers were calculated by linear regression (concentration versus area).

Instrumentation and chromatographic conditions.— HPLC was performed on an Alliance W 2690 separation module (Waters, MA, USA). Simultaneous monitoring of the OGAs was carried out with a Waters Refractive Index Detector R-2410 at 35 °C and a Shimadzu UV/VIS Detector SPD-10 AVvp at 235 nm. The separation was performed on an analytical scale (250 × 4.6 mm i.d.) Cyclobond I 2000 column and a guard column (20 × 4.0 mm i.d.) of the same material (both from Advanced Separation Technologies, Whippany, NJ, USA). The column was operated at a temperature of 40 °C. The elution was performed with two different isocratic systems: (1) ammonium formate (55 mM, pH 4):methanol (70:30, v/v) at a flow rate of 1 mL/min, (2) ammonium formate (100 mM, pH 4):methanol (70:30, v/v) at 0.8 mL/min. The injection volume for all samples was 50 μL. All eluents were filtered (0.2 μm) prior to HPLC analysis.

HPLC–MS was carried out on an HP HPLC series 1100 (Hewlett–Packard, Waldbronn, Germany) combined with a single quadrupole mass spectrometric detector Platform II (Micro Mass, Manchester, UK). Conditions were as follows: electrospray-ionization (negative mode), capillary voltage 3 kV, source temperature 120 °C, cone voltage ramp 24–69 V, multiplier voltage 650 V. The instrument was calibrated using a polyethylene glycol mixture up to m/z 2000, and mass spectra were acquired by scanning from m/z 170 to 2150. The flow was split 1:20 before entering the electrospray interface.

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