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Efficient purification of small unsaturated oligoglucuronides by reversed-phase chromatography

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Abstract

Ion-exchange chromatography has been applied to purification of unsaturated oligoglucuronans. After an isocratic elution on a strong anion-exchange column, the collected fractions were desalted by low pressure size exclusion chromatography. However, this efficient separation was limited by the time required to desalt. So, we developed a reversed-phase chromatography method using back ionization of oligomers. Two C18 columns were tested with trifluoroacetic acid (TFA 0.7%) as eluent. Different selectivities and column stabilities were observed in this acidic condition. The scale up for semi-preparative applications enabled us to recover pure unsaturated oligoglucuronans without desalting step.
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1. Introduction

Anionic polysaccharides such as those including uronic acids and/or sulphated functions in their structures have been intensively studied these last years for their biological, physiological [1–3] and rheological properties which are reinforced by their polyelectrolyte character [4]. Anionic polysaccharides were traditionally extracted and purified from animals (glycosaminoglycans), plants (polygalacturonic acid), *algae* (alginate, fucan) and microorganisms (polyglucuronic acid) but also came from modifications of neutral polysaccharides such as dextran or cellulose [1,2,5–9]. Moreover, it was shown that some oligosaccharides derived from these macromolecules exhibited specific properties as signalling molecules [10–12]. Generally, all these acidic oligomers resulted from the degradation of macromolecules by chemical, physical or enzymatic methodologies [11,13]. In this context, the non-availability of pure oligomers with specific degree of polymerization (dp) was restricting the comprehension of the relationship between structures and biological activities. Consequently, anionic oligosaccharide separation has been fully studied during the last decades, especially for oligogalacturonates, oligomannuronates and oligoguluronates. HPLC and high pressure anion-exchange chromatography (HPAEC) analytical strategies have been successfully employed and made it possible to separate anionic oligomers with dp up to 20 [14–19]. No real efficient semi-preparative chromatographic procedures have been published until now because anion-exchange chromatography at semi-preparative scale created some limitations. For example, high ionic strengths (commonly NaOH combined with a gradient of sodium acetate for HPAEC and salts for DEAE) were needed to elute oligomers. These high salt or sodium hydroxide concentrations ended up in very long and tedious desalting steps. An alternative to anion-exchange was size exclusion chromatography at low and high pressures. This method has been successfully investigated for the purification of oligoalginates but the elution time of each step was a limitating factor [20,21]. In addition, the anionic character of oligoalginates crea-

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ted a low resolution. Furthermore, desalting was often necessary after each separation. Another original method consisting in pseudobioaffinity chromatography has also been tested with acetylated oligoglucuronans. These acidic oligomers were purified at the milligram scale using L-histidine immobilized on PEVA hollow fiber membranes [22,23]. However, the chromatographic matrices are not available and a desalting step was also required. As a consequence, it is necessary to have methods to recover large quantities of pure oligouronides or other acidic oligomers in order to determine which part of the degree of polymerization is involved in biological activities. No problem is encountered with neutral oligomers which have been easily separated by semi preparative or analytical HPLC using for example reversed-phase chromatography [24,25]. In addition, eluents used with C18 columns can be easily evaporated under vacuum. So, our work was to remove the anionic character of oligoglucuronans by back ionization (full protonation of the carboxyl groups). This enabled us to purify them as neutral oligomers on reversed-phase chromatography. The oligomers chosen as a model were generated thanks to a fungal glucuronan lyase acting on a deacetylated form of glucuronan [26]. Native glucuronan is a bacterial homopolysaccharide composed of β-D-(1,4)-glucopyranosyluronic residues variably acetylated at C3 and/or C2 position. The unsaturated oligoglucuronans obtained after degradation were easily detectable during separation thanks to their UV absorbance (λ_{235}). They constituted thus a good representation of acidic oligomers. First, we purified oligoglucuronans with a traditional strong anion-exchange method at semi-preparative scale. Finally we developed a new method using reversed-phase chromatography at acidic pH.

2. Experimental

2.1. Production of deacetylated glucuronan

The *Sinorhizobium meliloti* M5N1CS mutant strain was grown at 30 °C in a 20 L bioreactor (SGI) with 15 L of rhizobium complete (RC) medium [27], supplemented with sucrose 1% (w/v) (RCS medium). The inoculum was a 1.5 L of RCS medium inoculated with *S. meliloti* M5N1CS, and was incubated at 30 °C for 20 h on a rotary shaker (120 rpm). After 72 h of incubation, broth was centrifuged at 33,900 × *g* and 20 °C for 40 min. After that, polysaccharides in the cell-free broth were precipitated by addition of three isopropanol volumes and collected by centrifugation (33,900 × *g*, 40 min, 20 °C). Pellets were then freeze-dried. The dry glucuronan was dissolved (4 g/L) in water and isopropanol precipitation step was repeated twice. Finally, glucuronan was deacetylated during 12 h at pH 11.8 (addition of KOH 2 M) and at 50 °C. The deacetylated glucuronan was purified by a similar isopropanol precipitation.

2.2. Production of crude enzyme extract

Trichoderma sp. GL2 was cultivated on 4 L of *Trichoderma* minimum medium supplemented with glucuronan (4 g/L) as single carbon source [26]. The strain grew at 25 °C on a rotary shaker (120 rpm) for 90 h.

The extracellular medium was collected by a step of vacuum filtration on fritted glass (150 μm), followed by filtration on a Millistak+ Mini MC0CH cellulose ester capsule (Millipore, Bedford, USA). Afterwards, the extracellular medium was concentrated down to 400 mL using an ultrafiltration device (normal molecular weight cut off (NMWCO): 5 kDa, 0.1 m^2 from Sartorius, Goettingen, Germany). A final concentration step was carried out on an Amicon stirred cell holding a 10 kDa polyethersulfone ultrafiltration disc (Millipore, Bedford, USA) down to a final volume of 50 mL.

Considering that one unit of activity corresponds to the release of 1 μmol of dp 3 oligoglucuronan (extinction coefficient = 4391 M^{-1} cm^{-1}) at 235 nm per minute, 1100 U of glucuronan lyase (GL) activity were collected.

2.3. Enzymatic degradation of deacetylated polymer

Deacetylated glucuronan was degraded at room temperature in water under gentle stirring using 12 U of crude enzyme per gram of glucuronan. After 30 min of incubation, reaction was stopped by heating the medium at 95 °C during 5 min. The medium was then centrifuged 15 min at 8000 × *g* to remove insoluble particles. Finally the pool of oligoglucuronans was freeze-dried.

2.4. Chromatography

Waters 600 and Waters LC4000 (Milford, MA, USA) systems were used for analytical and semi-preparative HPLC with Prevail C18 column and Nucleosil 100-5 SB column. Unsaturated oligoglucuronans were monitored using an UV detector (Waters 2487) at 235 nm. Chromatogram data acquisition was performed using Empower software.

Shimadzu system (LC-8A pumps and SCL-10A interface) was used for assays with the Nucleodur C18 Pyramid column. Unsaturated oligoglucuronans were monitored using an evaporative light scattering detector (ELSD 800 from Alltech). Gas pressure was equal to 3 bar and temperature to 50 °C. Data acquisition was performed using Class-VP software.

2.4.1. Anion-exchange chromatography

The Nucleosil 100-5 SB analytical (250 mm × 4.6 mm i.d.) and semi-preparative (250 mm × 21 mm i.d.) columns were purchased from Macherey Nagel (Düren, Germany). The columns were kept at room temperature. A semi-preparative pre-column (50 mm × 21 mm i.d.) was added for the purification at large scale. Analytical elution was run isocratically with $NaNO_3$ 200 mM at a flow rate of 0.8 mL/min. Sample concentration was 30 mg/mL. The sample injection volume was 20 μL. Semi-preparative elution was run isocratically with the same eluent at a flow rate of 13 mL/min. Five millilitres of samples with a concentration of 12 mg/mL were loaded on the column.

2.4.2. Reversed-phase chromatography

Two categories of columns designed especially for applications in eluent systems up to 100% water were employed at this stage.

Prevail C18 analytical (250 mm × 4.6 mm i.d., 5 μm particle size) and semi-preparative (250 mm × 22 mm i.d., 5 μm particle size) columns were from Alltech (Deerfield, IL, USA). Analytical injection volume was 100 μL of sample (10 mg/mL). Several eluents were tested with a flow rate of 1 mL/min: Milli-Q water (Millipore, Bedford, USA), trifluoroacetic acid 0.7% (pH 1.2), trifluoroacetic acid 0.4% (pH 1.4), trifluoroacetic acid 0.1% (pH 1.9) in isocratic or gradient mode. Semi-preparative injection volume was 2 mL of sample (30 mg/mL). The elution was performed with a flow rate of 17 mL/min by a gradient mode with trifluoroacetic acid 0.7% (eluent A) and water (eluent B). The gradient was 0% of B during 5 min, 100% of B for 55 min and finally equilibration step with 0% of B (20 min).

Nucleodur C18 Pyramid 5 μm analytical (250 mm × 4.6 mm i.d.) column was from Macherey-Nagel (Düren, Germany). Samples (20 μL) were loaded on column at 5 mg/mL. The elution was achieved with the eluents previously described and the following gradient: 0% of B during 2 min, 0–100% of B in 5 min, 100% of B for 10 min, 100–0% in 5 min and finally equilibration step with 0% of B (18 min).

2.4.3. Size exclusion chromatography

The fractions collected from oligoglucuronan separations with the Nucleosil 100-5 SB column were desalted with gel permeation chromatography on a Biogel P6 Fine (Bio-Rad, Hercules, USA) column (1 m × 26 mm) from Amersham Biosciences (GE Healthcare, Fairfield, USA). Milli-Q water at 0.8 mL/min was used for elution and 5 mL of samples were loaded on column.

2.5. Mass spectrometry

2.5.1. LC–MS

LC–MS analyses were performed on a Waters LC4000 system coupled with a single quadrupole mass spectrometer ZQ (Waters-Micromass, Manchester, U.K.) equipped with an electrospray ion source (ESI–MS).

The chromatography was carried out using the conditions described in Section 2.4.

The effluent was flow-split via a peek tee with 1/5 of the flow directed toward the ESI source of the ZQ instrument and the residual 4/5 directed toward the UV detector. LC–ESI–MS data were recorded in the negative ion mode. The source and desolvation temperatures were kept at 120 and 250 °C, respectively. Nitrogen was used as a drying and nebulising gas at flow rates of 450 and 100 L/h, respectively. The capillary voltage was −3.5 kV and a cone voltage range from −20 to −40 V was used (−ESI). Scanning was performed in the range 50–1550 Da at a scan rate of 1 s/scan. Data were collected in the continuous mode. Data acquisition and processing were performed with MassLynx V4.0 software.

2.5.2. HR-MS and MS–MS analyses

High resolution electrospray mass spectra (HR-ESI–MS) in the negative ion mode were obtained on a Q-TOF *Ultima Global* hybrid quadrupole/time-of-flight instrument (Waters-Micromass, Manchester, U.K.), equipped with a pneumatically assisted electrospray (Z-spray) ion source and an additional sprayer (Lock Spray) for the reference compound.

The samples were dissolved in Milli-Q water and the solutions were directly introduced (5 μL/min) through an integrated syringe pump into the electrospray source. The source and desolvation temperatures were kept at 80 and 150 °C, respectively. Nitrogen was used as the drying and nebulising gas at flow rates of 350 and 50 L/h, respectively. The capillary voltage was −3.5 kV, the cone voltage −80 V and the RF lens1 energy −45 V (−ESI). For collision-induced dissociation (CID) experiments, argon was used as collision gas at an indicated analyser pressure of 5×10^{-5} Torr and the collision energy was optimised for each parent ion (10–60 V). Lock mass correction, using appropriate cluster ions of an orthophosphoric acid solution (0.1% in H_2O/CH_3CN 50/50, v/v), was applied for accurate mass measurements. The mass range was typically 50–1050 Da and spectra were recorded at 1 s/scan in the profile mode at a resolution of 10000 (FWMH). Data acquisition and processing were performed with MassLynx 4.0 software.

3. Results

Two oligoglucuronan pools were prepared by enzymatic degradation of deacetylated glucuronan. An ESI–MS analysis of them led to the detection of four and six degrees of polymerization (dp) from 1 to 4 and 1 to 6 respectively. Their specific molecular weights $[M - H]^-$ were reported in Table 1. Thus these pools of unsaturated oligoglucuronans were employed as a model.

3.1. Purification of oligoglucuronans using anion-exchange chromatography

First, oligoglucuronans were resolved by anion-exchange chromatography in similar conditions than those previously described for oligoalginates [28]. In consequence Nucleosil 100-5 SB analytical column was used to develop successfully a separation method of an unsaturated oligoglucuronan mix with dp between 1 and 6. Then the purification was increased to a larger scale on a semi-preparative column at a flow rate of

Table 1
Specific molecular weights of unsaturated oligoglucuronan detected by ESI–/TOF-MS

Degree of polymerization (dp)	$[M - H]^-$
1	175
2	351
3	527
4	703
5	879
6	1055

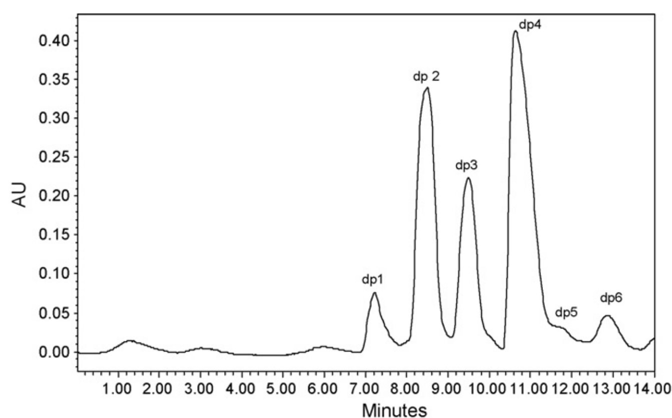

Fig. 1. Separation of unsaturated oligoglucuronans (60 mg) using semi-preparative anion-exchange column eluted with $NaNO_3$ 200 mM at a flow rate of 13 mL/min. The detection was achieved with an UV detector at 235 nm.

13 mL/min with $NaNO_3$ 200 mM as eluent (Fig. 1). Sixty milligrams of unsaturated oligoglucuronans were loaded on column without altering the high quality resolution of the separation in 15 min. As expected, six significative chromatographic signals were observed and assigned to oligoglucuronans with degrees of polymerization from 1 to 6. After this first step, the fraction corresponding to a supposed oligomer of dp 3 was freeze-dried and loaded on size exclusion chromatography (Biogel P6) to release nitrate sodium and to evaluate the efficiency of pure oligosaccharide recovery. After a final freeze-drying step, 3 mg of a compound were weighted and identified as a dp 3 oligoglucuronan by ESI–MS ($[M-H]/z=527$). The yield was 5% of the initial mass loaded on the strong anion-exchange column. So the release of salts by size exclusion chromatography was considered as a limiting step with regard to time of elution (11 h) and to low yields.

3.2. Purification of oligoglucuronans using reversed-phase chromatography

According to the poor desalting step efficiency after anion-exchange chromatography, another method was considered. The aim consisted to apply reversed-phase chromatography techniques generally used to purify neutral oligosaccharides. Oligouronides were dissolved in an aqueous acidic buffer three points bellow their pK_a (evaluated previously between four and five [23]) to acidify their carboxylic functions. In these conditions, the ionic character of oligoglucuronans disappeared. Thus it was possible to fractionate them according to hydrophobic interactions with a C18 phase. On the C18 Prevail column three trifluoroacetic acid concentrations (0.7, 0.4 and 0.1%, v/v) were tested as eluent. They corresponded to pH of 1.2, 1.4, and 1.7, respectively. The finest separation of an unsaturated oligoglucuronan mix with dp between 1 and 6 was obtained with the more acidic conditions thanks to an isocratic elution (Fig. 2). Each peak was characterized by mean of a chromatographic system coupled to an ESI/MS (data not shown). Only one oligomer was identified on each peak, except for dp 1 and 2. Moreover a partial separation of the two anomeric forms was observed. Nevertheless retention time of the dp 4 oligoglucuronan was above 40 min and broadened with this isocratic conditions. If we considered dp 5 and 6, the retention time was too long to consider a scale up of the method. In order to resolve these compulsions, several gradient configurations were tested using water and trifluoroacetic acid (0.7%). The most efficient gradient was 5 min trifluoroacetic acid 0.7%, 55 min water and 20 min trifluoroacetic acid 0.7%. Fig. 3 underlines that in these conditions, a mix of dp 1–6 may easily be eluted. All peaks were identified thanks to LC–ESI–MS coupling. The retention time of a dp 4 oligoglucuronan decreased to 22 min in comparison with the previous isocratic conditions.

With regard to the purification efficiency obtained with the analytical column, a semi-preparative separation was undertaken. 2.5 g of a dp 1–6 mix were loaded on a semi-preparative Prevail C18 column (22 mm i.d.) with the gradient established previously and a flow rate of 17 mL/min. Each fraction was collected and concentrated by evaporation under vacuum at 30 °C. After the concentration step, fractions were freeze-dried.

Purity of fractions B and C was confirmed thanks to a high resolution mass spectrometry analysis (Fig. 4). Spectrum of fraction B pointed a single ion ($[M-H]^-$) at $m/z=527$ corresponding to an oligoglucuronan of dp 3. The fragmentation via MS–MS revealed ions matching with the dp 1 and dp 2 residues ($m/z=175$ and $m/z=351$). Spectrum of fraction C revealed also a single ion ($[M-H]^-$) at $m/z=703$ corresponding to an oligoglu-

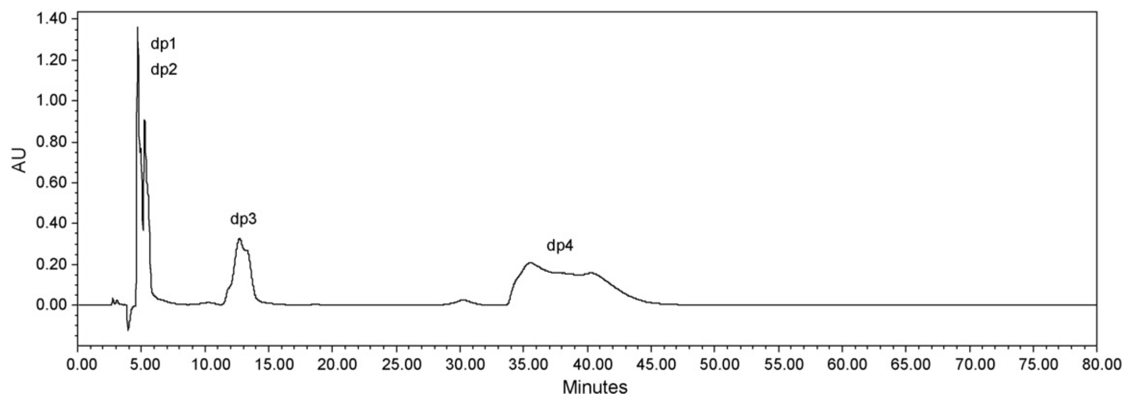

Fig. 2. Separation of unsaturated oligoglucuronans on analytical Prevail C18 column eluted at 1 mL/min by TFA 0.7% in water. The detection was achieved with an UV detector at 235 nm.

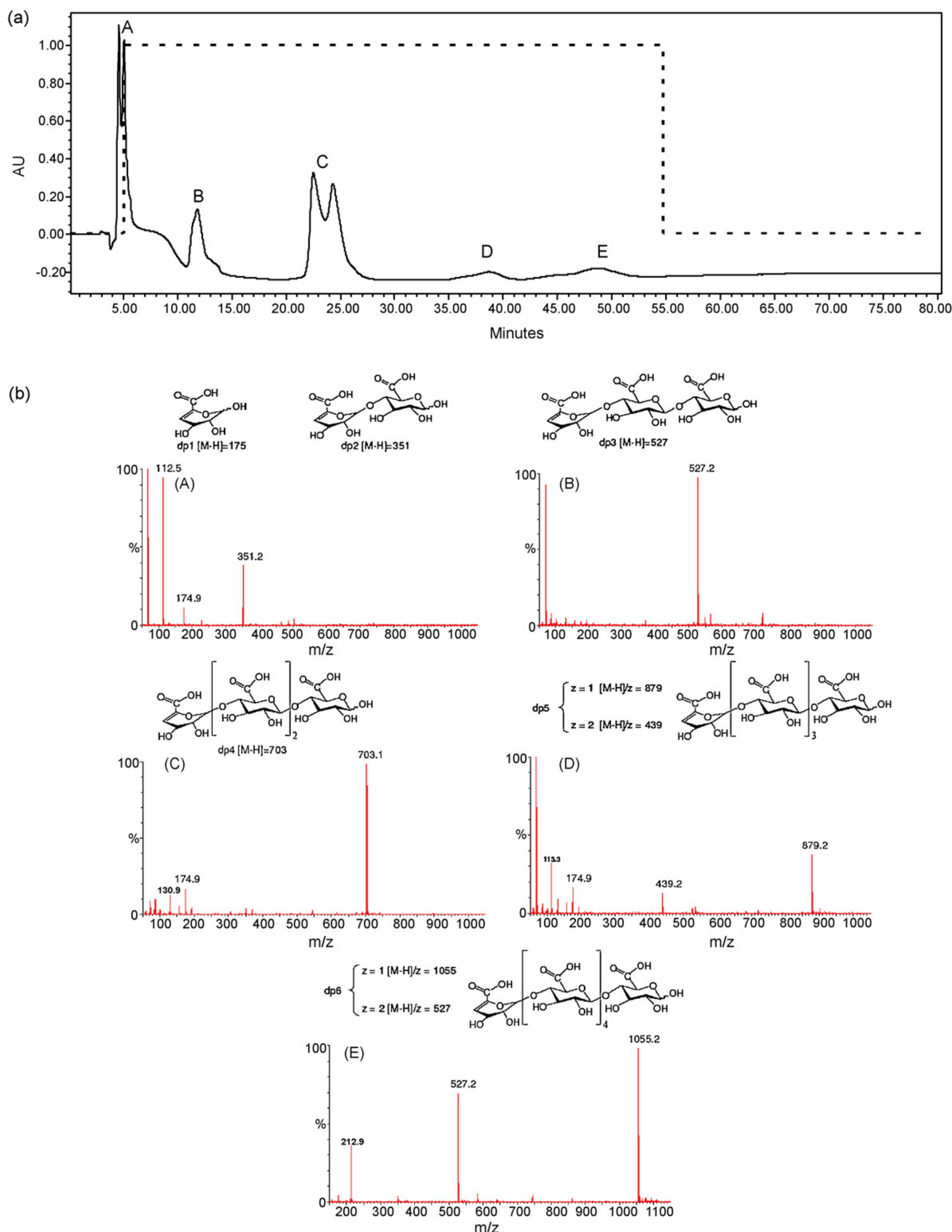

Fig. 3. LC–MS analysis performed with the analytical Prevail C18 column coupled with a single quadrupole mass spectrometer ZQ (ESI–MS) used as detector. (a) Chromatogram of unsaturated oligoglucuronan separation at analytical scale eluted by a gradient (- - -) of TFA in water (1 mL/min) between 0 and 100%. The detection was achieved with an UV detector at 235 nm and (b) ESI–MS of each fraction from (a) (A, B, C, D and E) in the negative ion mode.

curonan of dp 4. After fragmentation via MS–MS, ions matching with the residues from dp 1 to dp 3 ($m/z = 175$, $m/z = 351$ and $m/z = 527$) were detected. Table 2 shows the yields of oligoglucuronan recovery after elution of 2.5 g of oligoglucuronan mix. As seen on it oligouronides with dp 3 and 4 could be purified at the gram scale.

Nevertheless, the previous column was used under the manufacturer recommended pH (pH > 1.5) for the stability of the bonded phase. As a consequence another C18 stationary phase (Nucleodur C18 Pyramid) was implemented in similar conditions. This Nucleodur C18 Pyramid column was conceived for separation of compounds in water and in a large range of pH

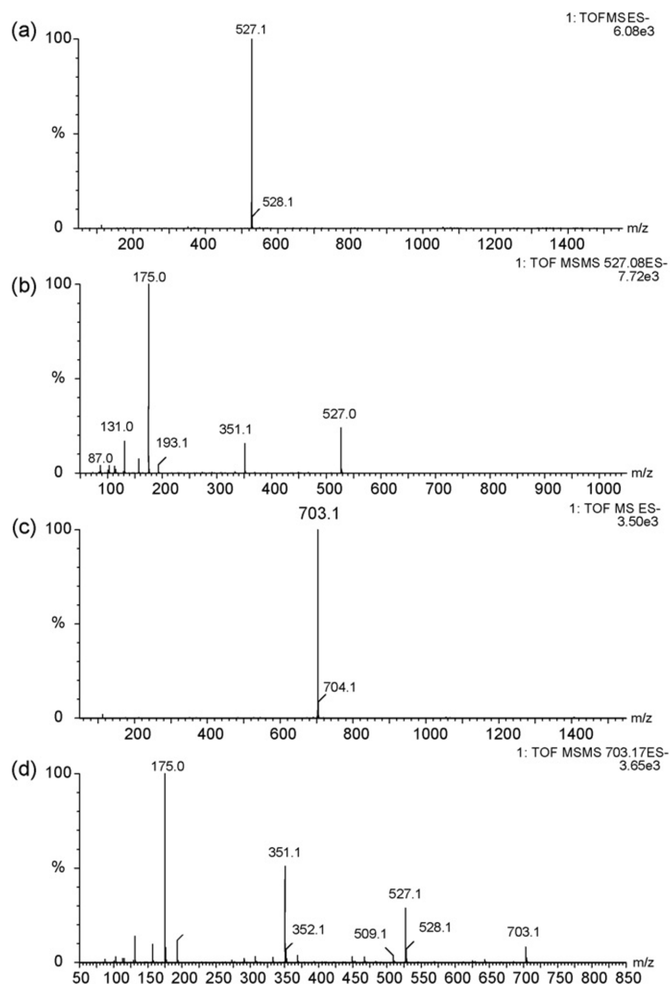

Fig. 4. High-resolution (HR) mass spectra of fraction B corresponding to the dp 3 (a) HR-MS, (b) HR-MS–MS) and fraction C corresponding to the dp 4, (c) HR-MS and (d) HR-MS–MS).

(1–9). Due to the trifluoroacetic acid absorbance at 235 nm, UV detector was replaced by an ELSD detector to avoid the gradient impact on the baseline.

After improving the gradient with an unsaturated oligoglucuronan mix with dp between 1 and 4, Nucleodur C18 Pyramid column gave a better resolution than the Prevail C18 column. Contrary to the previous purification, none of the peaks broadened (Fig. 5). The stability of the column was tested during 50 injections without observing any loss in the resolution and any pressure increase. As a result the phase stability in these drastic elution conditions was validated.

Table 2
Material balance of unsaturated oligoglucuronans from purification on a semi-preparative Prevail C18 column

Fraction	Degree of polymerization (dp)	Yield (%)
A	1–2	29.2
B	3	14.9
C	4	39.2
D	5	1.2
E	6	4.1

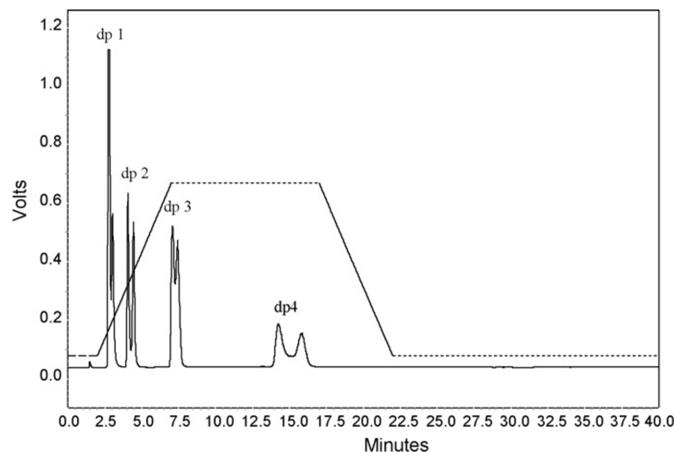

Fig. 5. Separation of unsaturated oligoglucuronans with the Nucleodur C18 Pyramid column eluted by a gradient of TFA in water (1 mL/min) between 0 and 100%. The detection was achieved with an ELSD detector.

4. Discussion

Two methods were presented here. They allowed to purify unsaturated oligoglucuronans from dp 1 to dp 4 obtained from glucuronan enzymatic degradation by a fungal glucuronan lyase. Purification using anion-exchange chromatography made it possible to separate each degree of polymerization in a short time with an isocratic elution. However, each collected fraction needed to be desalted. This step was carried out using permeation gel chromatography and required too much time at a semi-preparative scale (11 h were necessary to desalt 3 mg of oligomer). Thus it constituted the limiting stage because the ratio salts:unsaturated oligoglucuronans at each injection was 75:1. In this context, we developed a reversed-phase chromatography procedure. The principle of this separation was to purify unsaturated oligoglucuronans on reversed-phase chromatography using back ionization with an eluent at approximately three pH units less than oligoglucuronans pK_a ($4 < PK_{a_{oligomers}} < 5$). In these conditions unsaturated oligoglucuronans were totally acidified and interacted with the C18 phase as neutral oligosaccharides. Trifluoroacetic acid has been chosen because this acid could be easily removed using evaporation under vacuum.

Although unsaturated oligoglucuronan purification using Prevail C18 was longer than purification using Nucleosil 100-5 SB, TFA removal step was quicker than a desalting step. Consequently, global purification using reversed-phase chromatography was the quickest one.

However, we obtained best results using a Nucleodur C18 Pyramid column. Compared to the Prevail C18 column, this last stationary phase was manufactured to work in water with pH down to 1. Furthermore a better resolution was obtained and we managed to separate dp 1 from dp 2. Total elution time was reduced again.

Our results showed the possibility to use reversed-phase chromatography to purify unsaturated oligoglucuronans up to dp 4 with significant yields and good bonded phase resistance to drastic elution conditions. In the future we envisage to develop this method to other unsaturated oligouronides such as

oligogalacturonates or oligoalginates. Moreover the impact of oligosaccharide substituents such as acetates will be investigated in order to study the separation of oligomers with the same dp but different substitution degrees.

Reversed-phase chromatography by back ionization, while offering an interesting alternative to anion-exchange chromatography has only been employed for the analysis of relatively small unsaturated oligoglucuronans (dp < 5).

Acknowledgements

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